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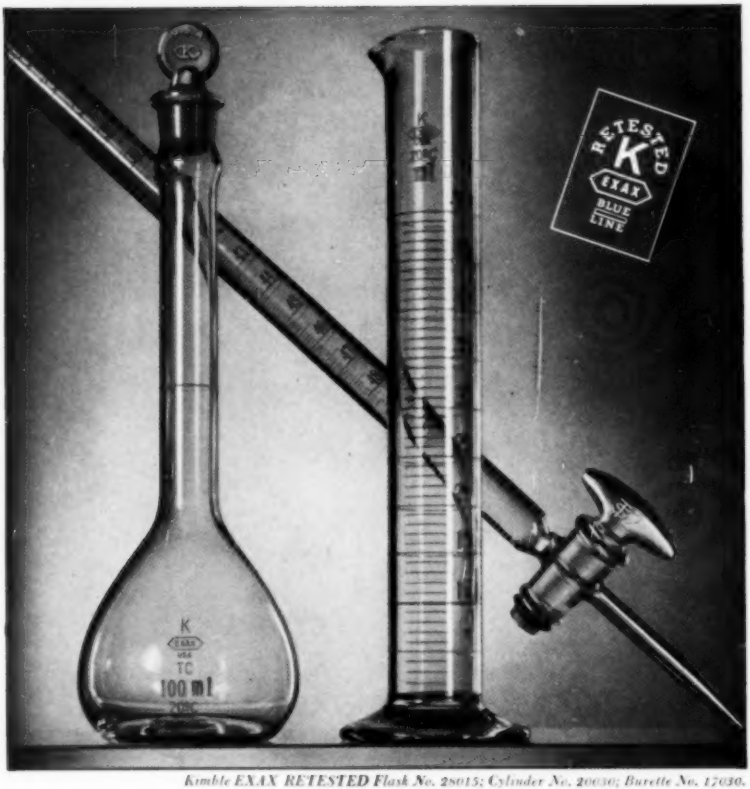

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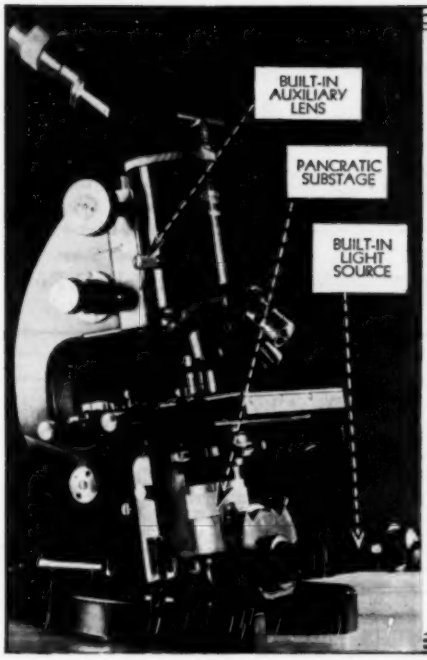

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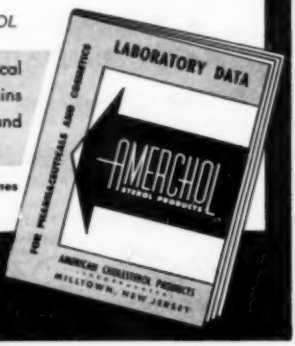

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VOLUME XXXVIII — *OCTOBER, 1949* — NUMBER 10
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The Identification of Some Autonomic Drugs by Means of the Optical Properties of the Diliturates*

By ELMER M. PLEIN†

The optical crystallographic properties of the diliturates (nitrobarbiturates) of *dl*-amphetamine (Benzedrine), *d*-amphetamine (Dexedrine), ephedrine, *dl*-ephedrine (Racephedrine), phenylpropanolamine (Propadrine), and *p*-hydroxy-*α*-methylphenethylamine (Paredrine) serve to identify each of the products.

THE USE OF the optical crystallographic properties of the diliturates has been suggested as means of identification of organic bases by the authors in previous papers (1, 2), in which they reported the optical constants for the diliturates of some primary aliphatic amines and some primary aromatic amines. The optical crystallographic identification of some amino acids with dilituric acid has been reported by Larsen, Witt, and Poe (3). The present communication reports similar constants for the diliturates of some organic bases used as drugs.

* Received April 30, 1949, from the University of Washington College of Pharmacy.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.
† The author wishes to thank Sharp and Dohme, Smith, Kline and French Laboratories, and The Upjohn Co. who supplied the amines for this study.

METHODS

The methods employed are similar to those used in the earlier works. In preparing the diliturates the amine or amine salt was dissolved in water and a hot aqueous solution of dilituric acid was added. The amine dilturate precipitated on cooling.

The diliturates were recrystallized from water one or more times. The identity and purity of the salts were established by determination of the nitrogen content by the Kjeldahl method modified to include nitro compounds. The deviation of the percentages found from those calculated did not exceed 0.2 except for *dl*-phenylpropanolamine, 0.39.

The optical data for the diliturates of the six amines are included in Table I. The usual orientation of the diliturates of *dl*-ephedrine and *p*-hydroxy-*α*-methylphenethylamine does not present a true value for any principal index, but the values can be determined from crushed crystals. The usual orientation of ephedrine dilturate permits the determination of two indices, alpha and gamma. The crystals of the other diliturates examined are so oriented that true values for alpha and beta can be determined.

The systems to which the various crystals belong are indicated in Column 2 by the letters, *O* and *T*—orthorhombic and triclinic, respectively.

TABLE I.—OPTICAL PROPETRIES OF SOME AMINE DILITURATES

Amine	System	Extinction Angle	Optic Sign	Refractive Indices			Elongation	Dispersion
				Alpha	Beta	Gamma		
dl-Amphetamine (Benzedrine)	O	Parallel	−	1.470	1.645	1.698	*	$v > \rho$
d-Amphetamine (Dexedrine)	O	Parallel	−	1.471	1.653	1.704	*	$v > \rho$
Ephedrine	O	Parallel	−	1.544	1.619	1.655	−	$\rho > v$
dl-Ephedrine (Racephedrine)	T	38	−	1.537	1.662	1.731	*	$v > \rho$
p-Hydroxy-α-Methylphenethylamine (Paredrine)	T	43	−	1.450	1.731	>1.785	+	$v > \rho$
dl-Phenylpropanolamine (Propadrine)								
Lath-Shaped	O	Parallel	−	1.461	1.678	1.708	−	$v > \rho$
Lamellar	O	Parallel	−	1.471	1.663	1.685	−	$v > \rho$

The diliturates of these amines are so flattened that when suspended in a liquid nearly all crystals assume the same or nearly the same orientation. In Table II some optical and crystallographic properties are recorded which were observed on the most frequently occurring orientations. The optical orientation designated as obtuse or optic normal indicates that a centered figure is observed in the usual position of the crystal. The term "inclined" indicates that the interference figure is not centered. *dl*-Ephedrine and *p*-hydroxy-α-methylphenethylamine in their usual orientations present refractive indices which cannot be satisfactorily determined within narrow limits. These indices are reported as variable. The lower index for *dl*-ephedrine dilturate is near 1.648 and the higher index is near 1.705. The low index of *p*-hydroxy-α-methylphenethylamine is near 1.465 and while the higher index appears variable, it is greater than 1.785.

show constant values. An asterisk is used to indicate the higher refractive index in an orientation unless a value is given for it. Observed crystal angles (determined microscopically) are indicated at the corner of the diagrams. Two forms of crystals are observed for *dl*-phenylpropanolamine dilturate, hence two sets of drawings are included to represent them.

Additional descriptions of some of the diliturates will facilitate identification of them. The dilturate of *dl*-amphetamine forms small crystals which macroscopically have the appearance of cotton. The crystals are thin and may be described as acicular. Polarization colors are dull, mostly gray. The principal indices of *dl*-amphetamine dilturate are close to those of *d*-amphetamine dilturate, but the crystals of the latter are much larger. The latter crystals show cleavage lines parallel to the long axis of the crystal and they are easily fractured. Polarization colors are bright.

TABLE II.—THE MOST SIGNIFICANT PROPERTIES OF SOME AMINE DILITURATES

Amine	Habit	Optical Orientation	Extinction Angle	Refractive Indices	
dl-Amphetamine (Benzedrine)	Acicular	Obtuse	Parallel	1.470	1.645
d-Amphetamine (Dexedrine)	Tabular	Obtuse	Parallel	1.471	1.653
Ephedrine	Lamellar	Optic normal	Parallel	1.544	1.655
dl-Ephedrine (Racephedrine)	Tabular	Inclined optic axis	Variable	Variable	Variable
p-Hydroxy-α-Methylphenethylamine (Paredrine)	Tabular	Inclined optic normal	Variable	Variable	>1.785
dl-Phenylpropanolamine (Propadrine)	Lath-Shaped	Obtuse	Parallel	1.461	1.678
	Lamellar	Obtuse	Parallel	1.471	1.663

In Fig. 1 are diagrams of the crystals showing front views as the crystals appear in their most frequently occurring orientations, side views, and in some cases top views. Side and top views were obtained by rolling the crystals in Canada balsam. Extinction angles were all measured between the long direction of the crystal in its particular orientation and the position of nearest extinction. Dotted lines indicate vibration directions and apparent refractive indices are recorded for those crystals which

Lath-shaped crystals of *dl*-phenylpropanolamine dilturate are most frequently smooth, but some of them exhibit faint cleavage lines. The lamellar form shows considerable cleavage and the crystals have an unfinished appearance. These crystals often occur in bundles which have to be broken up to yield the individual crystals.

The principal indices of *p*-hydroxy-α-methylphenethylamine dilturate all must be determined from crushed particles. Beta can best be identified from

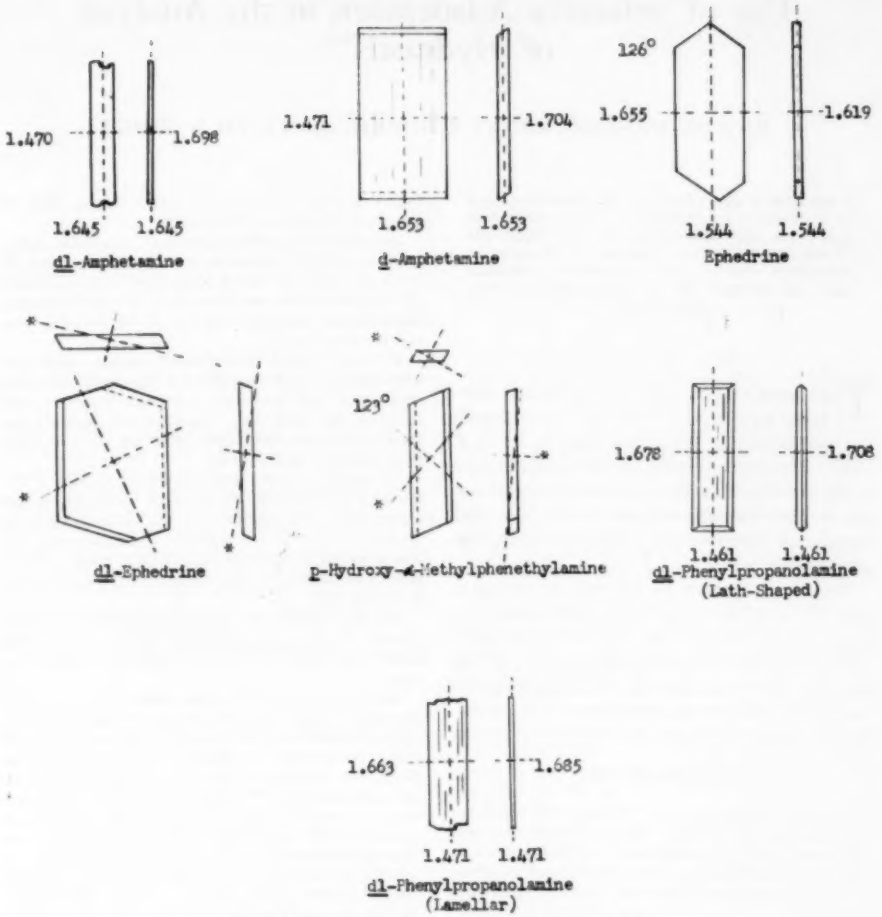

Fig. 1.—Diagrams of crystals of amine dilitrates.

those small particles which appear blue under crossed nicols.

dl-Ephedrine dilitrate forms very large yellow crystals which must be crushed and the indices identified from fragments. Most of the crystals give the appearance of being made up of crystals superimposed upon crystals. Interference figures are often double, but only one set of properties was found. Polarization colors are dull.

CONCLUSIONS

The dilitrates of the following amines have been prepared and their optical crystallographic properties determined: *dl*-amphetamine (Benzedrine), *d*-amphetamine (Dexedrine), ephedrine, *dl*-ephedrine (Racephedrine), phenylpropanolamine (Propadrine), and *p*-hydroxy-α-methylphenethylamine (Paredrine). These properties serve to identify the amines.

REFERENCES

(1) Plein, E. M., and Dewey, B. T., *Ind. Eng. Chem., Anal. Ed.*, **15**, 534(1943).
(2) Dewey, B. T., and Plein, E. M., *ibid.*, **18**, 515(1946).
(3) Larsen, L., Witt, N. F., and Poe, C. F., *Mikrochemie ver. Mikrochim. Acta.*, in press.

Use of Selective Adsorption in the Analysis of Hydrastis*,†

By JOSE MENDEZ‡, ERNST R. KIRCH, and RALPH F. VOIGT

A method is described for the determination of hydrastine in hydrastis. This method is based on the adsorption of the hydrastis alkaloids on Florisil columns. Hydrastine is selectively eluted with ammoniacal alcohol and determined by a spectrophotometric procedure.

THE FIRST official assay of hydrastis, introduced into the U. S. P. in 1910, is essentially the same procedure as that now found in the N. F. VIII. The difficulty with the official method lies in the fact that the ether-separated extracts contain not only the desired hydrastine but also small amounts of berberine and other extractives, non-alkaloidal in nature.

Recent applications by Brown and her associates (1) and Lasslo (2) of the principles of selective absorption to the problem of the separation of alkaloids have been successful. It was therefore deemed desirable to investigate the feasibility of isolating hydrastine on a quantitative basis by the use of an adsorption column.

EXPERIMENTAL

In 1938 Neugebauer and Brunner (3) indicated that alumina would only slightly adsorb the alkaloids from alcoholic preparations of *Hydrastis canadensis* L., *Berberis vulgaris* L. and *Mahonia aquifolia* L., and that the alkaloids could be eluted with 90% alcohol.

We investigated the adsorption characteristics of activated alumina,[1] using solutions of berberine (Merck & Co.) and hydrastine (Penick & Co.) in ether. Both of these alkaloids were completely adsorbed within certain limits. However, a quantitative separation could not be accomplished, since it was found that the 90% alcohol as well as other solvents would elute both of the alkaloids. Separation of these two substances therefore could not be effected by the use of activated alumina.

Adsorption columns employing fuller's earth, magnesium carbonate, magnesium oxide, or mixtures of magnesium oxide with Celite 501[2] were found to be of no practical value, due to their incomplete adsorption of alkaloids.

Investigations by Sussman, *et al.*, (4), on the industrial recovery of alkaloids by ion exchange, and the work of Lasslo (2) and of Kingsbury and co-workers (5) suggested the possible use of synthetic magnesium silicate (Florisil)[3] for the separation of hydrastine from berberine.

We have found that Florisil columns activated with ammonium hydroxide completely adsorb both hydrastine and berberine from acid-aqueous solutions of the alkaloids. Ammoniacal alcohol selectively elutes the hydrastine, but not the berberine, from columns so prepared.

No satisfactory method for the estimation of hydrastine is available. Volumetric methods for this assay have been employed in the German Pharmacopœia (6) and in the Swedish Pharmacopœia (7). These methods have given irregular results, as reported by Wales (8), by Eiderman (9), and by van den Berg (10).

We have found two poorly defined breaks in the titration curves for hydrastine solutions. No satisfactory end point could be calculated from these data.

Lyons (11) reported several color reactions which developed upon the addition of various oxidizing agents to solutions of hydrastis alkaloids in sulfuric acid. Hydrastine developed a transient brown-to-green color in the presence of potassium dichromate and sulfuric acid. We have investigated the possibility of adapting this reaction to the quantitative estimation of hydrastine. Experiments were carried out to investigate the following variables: (1) Increasing and decreasing the amount and concentration of potassium dichromate and of sulfuric acid. (2) Mixing the two reagents first and then adding the mixture to the hydrastine solution. (3) Adding dichromate solution to hydrastine first, followed by the acid, or reversing this procedure.

Comparison of spectrophotometric readings indicated that the best procedure was to add the dichromate to the solution of hydrastine, followed by the concentrated sulfuric acid. The color so produced reaches a maximum within five minutes and is stable for at least twenty hours. Spectrophotometric data on these solutions indicate maximum absorption at 600 mμ. At this wavelength, the color intensity of these solutions follows Beer's law as indicated in Fig. 1.

Berberine solutions treated with potassium dichromate and sulfuric acid produce the same color reaction as that described for hydrastine solutions. We have sought, therefore, a method for the determination of hydrastine in the presence of berberine

Solutions of berberine salts exhibit maximum absorption at 430 mμ. Berberine can be estimated

* Received April 30, 1949, from the Department of Pharmacognosy and Pharmacology and the Department of Chemistry, College of Pharmacy, University of Illinois.

† Abstract of a thesis submitted by Jose Mendez to the Graduate College of the University of Illinois in partial fulfillment of the requirements for the degree of Master of Science in Pharmacognosy, 1948.

‡ Fellow of the American Foundation for Pharmaceutical Education, and the University of San Carlos, Guatemala.

[1] Grade F-20 Mesh 80, Aluminum Ore Co., East St. Louis, Ill.

[2] Celite No. 501, Johns-Manville Co.

[3] Florisil, 60-100 Mesh, Floridin Co., Warren, Pa.

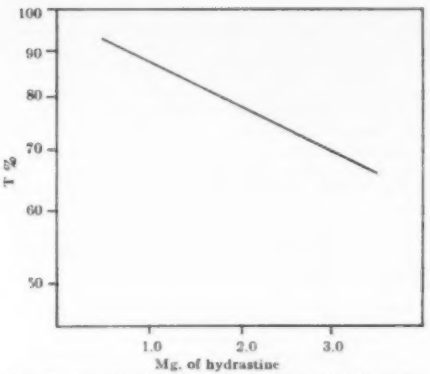

Fig. 1.—Standard curve for solutions of hydrastine.

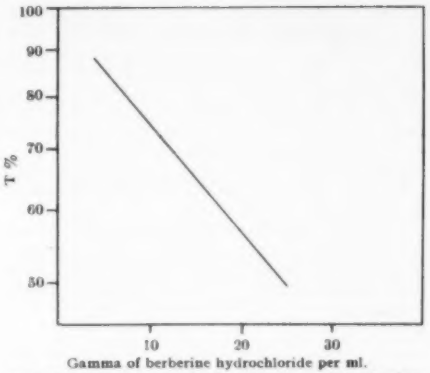

Fig. 2.—Standard curve for solutions of berberine.

quantitatively at this wavelength, as indicated in Fig. 2, and the presence of hydrastine does not interfere in this determination.

Our proposed method for the assay of hydrastine in hydrastis is therefore based upon the following principles: (1) Separation of hydrastine from berberine by adsorption on Florisil columns, with selective elution with ammoniacal alcohol. (2) Spectrophotometric estimation of the color produced in hydrastine solutions by oxidation with potassium dichromate and sulfuric acid.

The following data support the validity of this proposed method.

Reagents

Florisil, 60–100 mesh, Floridin Co., Warren, Pa.
Ammonia T. S., U. S. P.
Ethyl oxide, U. S. P.
Sulfuric acid, approximately 0.1 *N*.
Sulfuric acid, approximately 0.5 *N*.
Potassium dichromate, C. P. 1% solution.

Ammoniacal alcohol, a saturated solution of ammonia in 95% alcohol, prepared by passing ammonia gas into the alcohol. Final concentration about 10% NH_3 as determined by titration with 0.1 *N* acid.

Hydrastine (Penick & Co.), purified as follows: The alkaloid was dissolved in 1% sulfuric acid, the resulting solution made alkaline with ammonia T. S. and extracted with ether. The ethereal solution was evaporated to dryness and the residue was recrystallized from 95% alcohol. The product so obtained melted at 146° which is in agreement with the m. p. of 145° reported by Dott (12).

Adsorption Column.—Pyrex adsorption tubes (19 mm. diameter × 20 cm. height) with standard tapered glass joint are used. With the aid of a two-hole rubber stopper, the tube is inserted at the top of a small percolator equipped with a glass stopcock which permits the removal of quantities of collected liquid. Through the second hole of the stopper is attached a controlled vacuum line. In the upper end of the adsorption tube is placed a 250-ml. separatory funnel, as a reservoir. A rubber tube, attached to a glass tube inserted in the rubber stopper and provided with a pinch clamp permits the adjustment of the level of the solution to be adsorbed. A pad of glass wool is fitted into the bottom of the adsorption tube.

The Florisil is then packed to a height of about 10–13 cm., with sufficient pressure to insure a uniform column. A layer of glass wool is placed at the top of the column. One hundred milliliters of ammonia T. S. is drawn through the column with the aid of suction in order to activate the Florisil. The excess ammonia is removed by the use of 200 ml. of distilled water. The column is then ready for adsorption.

Extraction of the Crude Drug.—The official method in the N. F. VIII is followed in detail to the point where a measured quantity of the ethereal extract is taken for analysis.

Purification of the Alkaloids.—An accurately measured aliquot of the ether extract (25 ml. representing one-fourth of the weight of the hydrastis taken) is transferred to a 250-ml. beaker. Ten milliliters of approximately 0.5 *N* sulfuric acid are added and the ether is then completely removed by evaporation on a water bath. The resulting acid solution of the alkaloids is filtered through glass wool, and passed through the prepared adsorption column. The beaker is rinsed repeatedly with several portions of distilled water totaling 300 ml. and each rinsing is passed through the column. This is followed by 50 ml. of ether in order to replace most of the water in the column. Suction is continued during this procedure until the water-ether interface has descended to within 2 cm. of the bottom of the column. The ether is drained off and saved. One hundred milliliters of ammoniacal alcohol are then passed through the column and this eluate is combined with the ether fraction in a 250-ml. beaker. The liquid in the beaker is evaporated to dryness on a water bath.

Determination of Hydrastine.—The residue from the above evaporation is dissolved in 10 ml. of approximately 0.1 *N* sulfuric acid by heating on a water bath. This solution is transferred to a 50 ml. volumetric flask, completing the transfer with an additional 10 ml. of 0.1 *N* sulfuric acid and sufficient distilled water to make 50 ml.

Aliquots of this solution (usually 1–2 ml.) representing from 1 to 3 mg. of hydrastine are transferred to a 25-ml. volumetric flask. Four milliliters of 1% potassium dichromate solution are added, followed by 5 ml. of concentrated sulfuric acid. This mixture is thoroughly shaken and allowed to stand

for five minutes in order to develop the maximum color. Distilled water is cautiously added to within a few milliliters of the mark and the final volume is adjusted at 20°. Transmittance is read in a spectrophotometer at 600 mμ, against a reagent blank, and the corresponding values for hydrastine are calculated with the aid of the standard curve in Fig. 1.

Any berberine present in the eluate aliquot is estimated directly by means of spectrophotometric readings at 430 mμ, with the aid of the standard curve in Fig. 2.

Using these procedures, 8 assays were carried out on a sample of hydrastis. Findings so obtained were compared with the results of 6 assays of the sample by the official method. Results obtained are shown in Table I.

TABLE I.—COMPARISON OF THE RESULTS OBTAINED BY THE NATIONAL FORMULARY METHOD AND THE PROPOSED METHOD, IN 8 ASSAYS OF 1 SAMPLE OF HYDRASTIS

Alkaloids, N. F. Method, %	Alkaloids, Proposed Method, %		
	Hydrastine Plus Berberine	Berberine	Hydrastine
2.67	2.57	0.05	2.52
2.64	2.53	0.05	2.48
2.68	2.56	0.05	2.51
2.66	2.57	0.04	2.53
2.71	2.61	0.06	2.55
2.66	2.58	0.05	2.53
	2.58	0.08	2.50
	2.54	0.06	2.48
Av. 2.67	2.57	0.055	2.51

DISCUSSION

The proposed method for the estimation of hydrastine represents a distinct improvement over the official procedure. Gravimetric methods are time-consuming, and the final weight of "ether-soluble alkaloids" represents hydrastine plus some berberine and nonalkaloidal extractive.

The findings of the Subcommittee on Botanical Drugs of the American Drug Manufacturers Association (13) indicate that the extraction procedure described in the official assay effects a complete removal of the alkaloids of hydrastis. We deemed it desirable therefore to follow these directions to the point where the ether extract is obtained after standing overnight. This extract contains the hydrastine with other ether-soluble extractives. In the procedure in general use the well-known "shaking out" method is employed in order to obtain a solution of the alkaloid to be determined, free from interfering materials.

The procedure proposed not only simplifies the process of purification of the alkaloid hydrastine but increases the specificity of the method and effects a saving of time. Results obtained by the proposed procedure are lower than the results of the official assay on the same sample of hydrastis. The difference represents the other ether-soluble extractives included in the official assay.

The average amount of the alkaloid hydrastine in the sample of hydrastis was found to be 2.51% as compared to 2.67% when determined by the official N. F. Method.

The colorimetric method for the determination of hydrastine is to be preferred because of the uncertainty of the end point when solutions of hydrastine are titrated. The interference due to berberine which gives the same color reaction is not serious, because the yellow color of this alkaloid permits its spectrophotometric estimation at 430 mμ. By difference the amount of hydrastine can be calculated. In general such a procedure is not necessary. The Florisil column adsorbs both of the alkaloids, but only hydrastine is eluted by ammoniacal alcohol. The berberine remains in the column, and does not appear in significant amounts in the eluate, as is shown in Table II.

TABLE II.—RECOVERY OF HYDRASTINE AND BERBERINE

Hydrastine Added,[a] Mg.	Amount of Solvent Used in the Elution	Berberine Recovered, %	Hydrastine Recovered, %
50.0	50 ml. ether and 100 ml. ammoniacal alcohol	0.0	100.3
50.0		0.0	100.3
52.2		0.0	102.0
50.1		0.0	100.5
50.0		0.0	100.3
50.2		0.0	100.2
50.3		0.0	100.0
50.2		0.0	100.9
49.9		0.0	99.6

[a] 50 mg. of berberine hydrochloride were added to each amount of hydrastine.

Complete adsorption of alkaloids on the columns is evidenced by the absence of a precipitate on the addition of Valser's reagent to the filtrate.

SUMMARY

The separation of hydrastine from berberine has been accomplished by selective adsorption and elution of these alkaloids, using columns of activated Florisil.

A colorimetric procedure for the estimation of hydrastine so separated is described.

Results obtained by the proposed method are slightly lower than those obtained by the official method. The difference represents the berberine and nonalkaloidal ether-soluble extractives which are included in the official determination.

REFERENCES

(1) Brown, N. G., Kirch, E. R., and Webster, G. L., THIS JOURNAL, **37**, 24(1948).
(2) Lasslo, A., Thesis: "The Assay of Ipecac and the Purification of the Ether-Soluble Alkaloids with the Aid of an Adsorption Column," University of Illinois, Chicago, 1948.
(3) Neugebauer, H., and Brunner, K., *Pharm. Zentralhalle*, **79**, 161(1938).
(4) Sussman, S., Mindler, A. B., and Wood, W., "Recovery of Alkaloids by Ion Exchange," The Permutit Co., New York.
(5) Kingsbury, A. W., Mindler, A. B., and Gilwood, M. E., "Recovery of Nicotine by Ion Exchange," The Permutit Co., New York.
(6) "Deutsches Arzneibuch," 6 Ausgabe, 1926. R. V. Decker's Verlag, Berlin, 1926.
(7) "Sweden Pharmacopoeia X," Kungl. Boktryckerist., P. A. Norstedt & Söner, Stockholm, 1925, p. 171.
(8) Wales, H., *Ind. Eng. Chem.*, **18**, 390(1926).
(9) Eiderman, E. M., *Farm. Zhur.*, 602(1928).
(10) van den Berg, J., *Pharm. Weekblad*, **56**, 1124(1919).
(11) Lyons, A. B., *Western Druggist*, **8**, 73(1886).
(12) Dott, D. B., *Pharm. J.*, **109**, 607(1922).
(13) Copley, D. M., *Bull. Nat. Formulary Comm.*, **13**, 125 (1945).

Cultivation Studies of the Solanaceous Drugs. I. Post-Harvest Alkaloid Movement in Belladonna and *Datura* Species*,†

By W. R. BREWER‡ and L. DAVID HINER§

A series of experiments was carried on over a period from 1942 to 1947 involving belladonna and three species of *Datura* to determine the value of curing leaves while intact with the rest of the plant. The significance of the results was tested statistically. The tests showed that the drying of leaves intact on the plant increases their alkaloidal assay over the leaves which were dried separately; that the effect is not due to the position of the plant during drying or to gravity; that the enriching of the leaf with alkaloids is accompanied by a decrease in the alkaloids of the root or stem, which suggests relocation; and that the effect occurs in at least four species of solanaceous plants.

FOR MANY years, herbalists and herbal publications have advocated the curing of medicinal leaves by suspending the entire, excavated plants, top downward, in dry sheds from cords tied around the stems or roots. In a series of studies carried on in Ohio during 1942 with the drug, *Atropa belladonna*, L., the plants were cured at first by separating the fresh leaves from the stems and drying the two parts, thus separated, on trays. Later, due to the dwindling supply of labor available during this period a more rapid, labor-saving method was employed. The stems were cut near their bases and the plants were tied to, or were impaled on, nails driven through long wooden racks. In this way, they were cured hanging upside down and the leaves were stripped from the stem after drying. When the assays of these various products were compared, an indication was found that when whole plants were hung up to cure, there was a greater percentage of alkaloids in the leaves dried intact on the stem than in the leaves dried on the trays, separated from stems. Also, the stems of the plants cured intact were found to have a lower assay of alkaloids than those cured separated from their leaves.

In order to confirm these results and investigate the passage of alkaloids from the stems into the leaves, further studies were developed in 1943 and concluded in 1947.

Authentically labeled seed samples of *Atropa belladonna*, L., *Datura stramonium*, L., *Datura inermis*, Jacq., and *Datura tatula*, L., were obtained in 1942 from the research divisions of S. B. Penick & Company of New York City, Eli Lilly & Company of Indianapolis, and from the Bureau of Plant Industry of the United States Department of Agriculture at Beltsville, Md. Mature plants in the flowering stage were authenticated macroscopically by taxonomists of the horticulture and botany departments of Ohio State University as a double check.

EXPERIMENTAL

Plots of 30 plants each, of both one- and two-year-old individuals, of *A. belladonna* were harvested to provide leaf and stem parts which were cured separated on drying trays and also parts which were cured intact by impaling the whole plant on racks and separating leaf from stem after drying. Results of the assays of this material are shown in Table I. (All assays in this experiment were carried out according to the official method of the U. S. P. XII.)

TABLE I.—ALKALOIDAL ASSAYS OF BELLADONNA LEAF AND STEM AFTER SEPARATED AND INTACT CURING

	Separated, %		Intact, %	
	Leaf	Stem[a]	Leaf	Stem[a]
	0.32	0.50	0.47	0.35
	0.34	0.53	0.38	0.26
	0.33	0.48	0.38	0.34
Av.	0.33	0.50	0.41	0.32

[a] Part of root attached.

Since the roots of belladonna have been known to contain a higher percentage of alkaloids than other portions of the plant, it was believed that the high content could be transferred to the leaves by hanging the whole plant, including the roots, tops downward, from the drying racks. A total of 60 plants in two adjacent plots were harvested by excavating the whole plants. Those from one plot were cured by hanging the entire plant, including roots, stems, and leaves, tops downward from drying racks. Those from the other plot were cured by drying their roots, stems and leaves in three different drying trays. The results of the assays of this material are shown in Table II.

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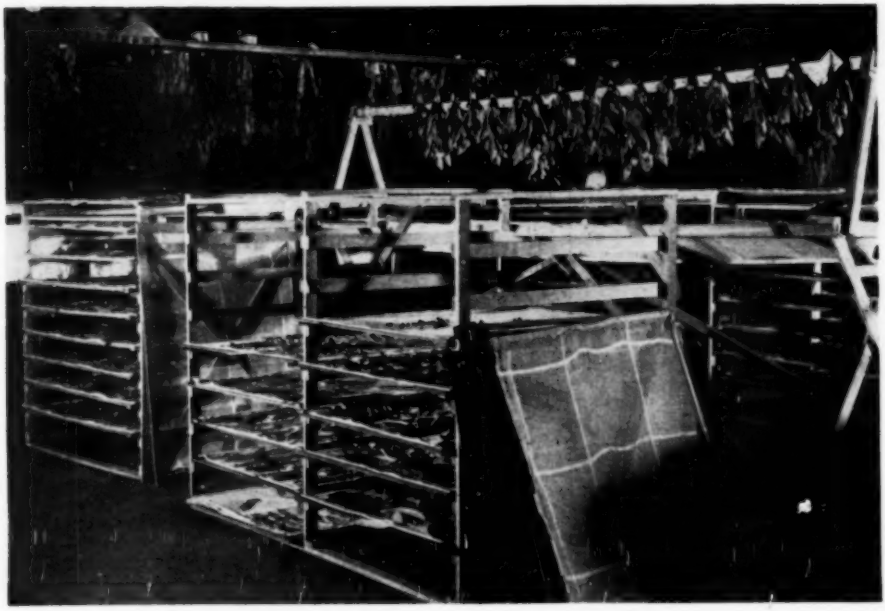

Fig. 1.—Drying racks used for curing belladonna.

These figures offer evidence of alkaloid movement. To determine the reliability of this evidence, a statistical test was made of the difference between the means of the above assays of leaves separated before curing and of leaves cured before separation. The tabulation of the leaf assays, their means, and the statistics involved in their testing appears in Table III.

The last line of Table III shows a probability of less than 1% in an experimental sampling of the extent described and that the difference in the mean assays could be as large as it is by chance. Such a small probability offers significant evidence that the difference in assay means is due to the method of curing and not due to chance, and that drying leaves intact on the plants increases their alkaloidal assay.

TABLE II.—ALKALOIDAL ASSAYS OF BELLADONNA LEAF, STEM, AND ROOT AFTER SEPARATED AND INTACT CURING

Separated, %			Intact, %		
Leaf	Stem	Root	Leaf	Stem	Root
0.36	0.19	0.61	0.42	0.17	0.47
0.34	0.16	0.50	0.43	0.22	0.35
Av. 0.35	0.175	0.555	0.425	0.195	0.41

TABLE III.—POST-HARVEST ALKALOID MOVEMENT IN LEAVES CURED, SEPARATED, AND INTACT

Assays in Parts per 10,000	
Leaves Separated Before Drying on Trays	Plants Dried on Racks, Then Leaves Separated
32	47
34	38
33	38
36	42
34	43
Mean 33.8	Mean 41.6

t = 4.29
$D.F.$ = 8
P = Less than 1%

A similar experiment was conducted in 1943 using plants in three different stages of development. Although roots were present on the plants which were cured intact, only leaf and stem samples were assayed. Results of the assays appear in Table IV.

From this table, it appears that the post-harvest mobility of belladonna alkaloids increases with increasing age of the plants beginning with plants in the preflowering stage and including time through the fruiting stage of development.

Two explanations seemed possible for the phenomenon of alkaloid movement in plants hanging upside down: (1) that some physiological process was tending to draw the alkaloids down into the leaves, and (2) that the movement was an effect of gravity. To determine the value of this latter explanation,

TABLE IV.—POST-HARVEST ALKALOID MOVEMENT IN BELLADONNA AT VARYING STAGES OF DEVELOPMENT

Development Stage	Assays in Parts per 10,000 — Parts Cured Separately		Whole Plant Cured, Then Separated	
Immature	Green stems	37	Green stems	43
	Leaves	31	Leaves	33
Flowering	Green stems	39	Green stems	43
	Leaves	35	Leaves	42
Fruiting	Green stems	30	Green stems	40
	Leaves	30	Leaves	53

plants were cured while being maintained in an upright position. If the effect were due to gravity, the alkaloids should have moved into the roots. See Table V. The results indicate that neither the position of the plant nor gravity controls the movement of alkaloids into the leaves during the drying process.

TABLE V.—POST-HARVEST ALKALOID MOVEMENT IN BELLADONNA PLANTS CURED IN AN UPRIGHT POSITION

Plant Parts Cured Separately on Trays, %		Whole Plants Cured Upright Before Separation, %	
Roots	0.52	Roots	0.33
Stems	0.17	Stems	0.21
Leaves	0.35	Leaves	0.41

To aid in the positive establishment of the fact that alkaloids move within the plant during curing, a test was devised utilizing only one set of plants, thereby eliminating the confusing factor of natural variation in alkaloidal content of different sets of plants. One half of the leaves of one group of belladonna plants in 1943 and of two groups in 1947 were stripped from the stems and dried separately on trays while the other half of the leaves from these plants were left intact to cure on the plants hanging from racks. The assays of these two sets of leaves, their group means, and the statistics of the test for significance of a difference between means appear in Table VI.

TABLE VI.—POST-HARVEST ALKALOID MOVEMENT SHOWN BY ASSAYS OF BELLADONNA LEAVES CURED INTACT COMPARED TO ASSAYS OF LEAVES FROM THE SAME PLANTS CURED AFTER SEPARATION

Assays in Parts per 10,000 — Half of Plants' Leaves Dried After Separation	Half of Plants' Leaves Dried Intact, Then Separated
37	49
31	41
36	42
Mean 34.7	Mean 44.0

$t = 2.985$
$D.\ F. = 4$
$P = 2\%$

The 2% probability appearing in the last line of the table indicates, significantly, that the half of the plants' leaves cured intact assayed higher than the other half cured separately—by a difference which is due to the treatment and not to chance. In 1947, a similar experiment with 30 plants each of three different species of the genus, *Datura*, was designed to replicate the "half-of-leaves" test on belladonna described in Table VI. The leaf assays, group means, and statistics appear in Table VII.

TABLE VII.—POST-HARVEST ALKALOID MOVEMENT IN THE "HALF-OF-LEAVES" TEST WITH *Datura* SPECIES

Species	Assays in Parts per 10,000 — Half of Leaves Dried After Separation	Half of Leaves Dried, Then Separated
D. stramonium, L.	53	61
D. inermis, Jacq.	49	59
D. tatula, L.	59	62
Mean	53.7	60.7

$t = 2.306$
$D.\ F. = 4$
$P = 4\%$

The probability of only 4% that the difference between group means could be as great as it is by chance, in the population from which the samples were taken, is considered to be significant evidence that the alkaloid was relocated in the intact leaves.

SUMMARY AND CONCLUSIONS

In order to investigate an observed difference in the alkaloidal content of leaves cured, separated, and intact on belladonna plants and, coincidentally, to determine the value of a traditional method of curing leaves by hanging whole herbs from rafters of drying sheds, leaves of belladonna and of *Datura* species were used in various curing studies over a period from 1942 to 1947. The results of the studies were subjected to statistical interpretation to determine their significance in the investigation.

The results of the various studies show:

1. That the drying of leaves intact on the plant increases their alkaloidal assay over the leaves which were dried separately.
2. That this effect is not due to the position of the plant during the drying procedure nor to gravity.
3. That the enriching of the leaf with alkaloids is accompanied by a decrease in the alkaloids of the root or stem, which is suggestive of relocation.
4. That the effect occurs in at least four species of solanaceous plants, *Atropa belladonna*, *Datura stramonium*, *Datura inermis*, and *Datura tatula*.

Procaine Penicillin and Sulfonamide Antagonism*

By H. FISCHBACH, H. WELCH, E. Q. KING, J. LEVINE, C. W. PRICE, and W. A. RANDALL

One and one-half million units (0.62 Gm.) of procaine penicillin were administered intramuscularly to each of 36 normal male adults. Three hours later samples of blood were collected and immediately analyzed for penicillin content and for procaine or its derivatives. It was found that a major portion of the procaine was converted to N-acetyl procaine. In seven instances out of 36 blood samples analyzed, low concentrations of *p*-aminobenzoic acid were detected. These results indicate that procaine penicillin may be administered with sulfadiazine, sulfathiazole, and sulfapyridine, but may be antagonistic toward sulfanilamide.

PROCAINE PENICILLIN was introduced in April, 1948, as a certified penicillin product in the form of a suspension of the salt in oil. Later other procaine penicillin preparations such as procaine penicillin for aqueous injection and procaine penicillin in oil with aluminum monostearate were developed. Prior to the certification of these drugs, consideration was given to the possible antagonistic action of the procaine portion of the molecule for the sulfonamides (1). This antagonistic action might be significant in those relatively infrequent occasions where the severity or type of disease might warrant the concomitant administration of both drugs. On theoretical grounds it was decided that the amount of procaine absorbed from the usual human dose of the relatively insoluble procaine penicillin in oil (300,000 units), would be so small that it would not exert any antisulfonamide action. However, since it was proposed to utilize as much as 1.5 to 2.4 million units of procaine penicillin in oil in a single injection in the treatment of early syphilis, it was thought desirable to undertake the present experimental work.

It has been shown (2) that when procaine is introduced into the body it is rapidly metabolized to other compounds, such as *p*-aminobenzoic acid and N-acetyl-*p*-aminobenzoic acid. Since N-acetyl-*p*-aminobenzoic acid has almost no antagonistic effect toward the sulfonamides, any *in vivo* antagonism may be ascribed almost entirely to free *p*-aminobenzoic acid. On the basis of *in vitro* studies Wyss, *et al.* (3), have reported that the molar ratios of several sulfonamides required to overcome the antagonistic action of *p*-aminobenzoic acid were of the following magnitude: sulfanilamide 4000–1, sulfaguanidine 4000–1, sulfacetamide 500–1, sulfapyridine 400–1, sulfadiazine less than 100–1, and sulfathiazole 50–1. Thus, if these *in vitro* ratios are the same *in vivo* it would take more than 1 γ of *p*-aminobenzoic acid per ml. of blood to antagonize the effect of 100 γ (10 mg. per cent) of sulfadiazine or sulfathiazole, but only 0.02 γ/ml. to neutralize the same amount of sulfanilamide.

* Received June 11, 1949, from the Federal Security Agency, Food and Drug Administration, Washington, D. C.

EXPERIMENTAL

Thirty-six normal male adults were injected intramuscularly with 1.5 million units of procaine penicillin (aqueous) representing 0.62 Gm. of procaine. Three hours later, samples of blood were collected and immediately analyzed for their penicillin content and for procaine or its derivatives.

Except for the recently described method of Brodie (2) for procaine, the existing chemical procedures for *p*-aminobenzoic acid were not sufficiently sensitive to be of value in the present study. In the method described herein, procaine and its metabolic products are separated into three groups by extraction with immiscible solvents at different *p*H values. Those fractions containing *p*-aminobenzoic acid derivatives are hydrolyzed to liberate free *p*-aminobenzoic acid which is determined by diazotization and coupling with N(1-naphthyl)ethylenediamine to form a highly colored derivative. Concentrations of less than 0.1 γ/ml. of *p*-aminobenzoic acid may be detected by this method.

Method.—To 5 ml. of the serum under test (if insufficient serum is available, make up to 5 ml. with water) add 6 ml. of isopropanol and 1 ml. of chloroform. Centrifuge to remove the precipitated protein. (NOTE: The relative volumes of the aqueous and the nonaqueous liquids are close to the limits of their mutual solubility, and occasionally two liquid phases will separate. When this occurs, the addition of 1 ml. of isopropanol will restore miscibility.) Transfer the clear supernatant to a separatory funnel and add 10 ml. of water. Add 5 drops of concentrated ammonium hydroxide and extract with two 35-ml. portions of chloroform. Filter the chloroform extract through a dry filter paper or a cotton plug into a flask and evaporate the solvent on a steam bath with a current of air impinging on the surface to prevent boiling or creeping (Fraction A). Strongly acidify the residual aqueous solution in the separatory funnel by the addition of 20 drops of concentrated hydrochloric acid and extract with two 35-ml. portions of chloroform-isopropanol (3 to 1 by volume). Filter and evaporate as above (Fraction B). To the remaining aqueous solution add 5 ml. of a 25% dibasic potassium phosphate

buffer solution and adjust the *p*H between 3.2 and 4.5 by the dropwise addition of ammonium hydroxide or hydrochloric acid solution, as needed. Extract with two 40-ml. portions of ether. Filter and evaporate as above (Fraction C). To each residue add 1 ml. of 1 *N* hydrochloric acid. Cover the flasks containing Fractions A and B with watchglasses and heat on a steam bath for fifteen minutes to hydrolyze the contents to *p*-aminobenzoic acid. Cool. To each add 1 ml. of 0.1% sodium nitrite solution. After one to two minutes add 1 ml. of 2% sulfamic acid solution. Shake occasionally for two minutes. Finally, add 0.5 ml. of a 0.1% aqueous solution of N(1-naphthyl) ethylenediamine dihydrochloride. At this stage the solutions will be cloudy due to suspended material extracted from the blood. To remove this, transfer the coupled solution to a small glass-stoppered test tube. Shake vigorously with a small volume (0.5 to 1 ml.) of chloroform and centrifuge to separate the phases. Draw off the clear supernatant aqueous layer with a drawn-out glass tube fitted with a rubber bulb and transfer it to matched tubes 10 by 100 mm. The color developed by the smaller amounts of *p*-aminobenzoic acid (less than 0.1 γ) may be detected visually in a good light against a white background. Prepare standards by adding known amounts of *p*-aminobenzoic acid or its derivatives to blank serum and subject them to the entire analytical procedure. The *p*-aminobenzoic acid content of the samples was estimated by visual comparison with the standard.

It must be pointed out that the identities of the components of the three fractions have been assumed from their behavior in the several extractions, rather than specifically proved by their isolation. There is ample evidence, however, as to the correctness of these assumptions. Fraction A will consist of only neutral or basic substances. Free procaine or N-acetyl procaine would be found here. In several preliminary experiments no free procaine was found. In a separate experiment, serum prepared from eight individuals treated with procaine penicillin was pooled and the alkaline extract corresponding to Fraction A was divided into two equal parts. One portion was diazotized and coupled after hydrolysis as described and the other portion treated without hydrolysis. An appreciable color was developed in the hydrolyzed portion, while no trace of color was developed in the nonhydrolyzed portion. This absence of free procaine is in accord with the findings of Brodie, *et al.* (2), who reported that no procaine was found in blood one hour after infusion of 2 Gm. of procaine. Since the common detoxication product of aromatic amines is the acetylated derivatives, the substance found in Fraction A is considered N-acetyl procaine.

In Fraction C will be found acids which contain a weakly basic group as shown by the fact that they may be extracted from aqueous solution at approximately *p*H 3.5, but not from alkaline or strongly acidic solution. Fraction B will be composed of acids in which this basic group is masked by a group which may be removed by hydrolysis. Brodie, *et al.* (2) intimate that *p*-aminobenzoic acid is conjugated as acetyl *p*-aminohippuric acid as well as *N*-acetyl *p*-aminobenzoic acid. These two compounds would be found in Fraction B. Analogously, it would be expected that *p*-aminohippuric acid would be found in Fraction C together with the free *p*-aminobenzoic acid. This would be significant if true, since Beyer, *et al.* (4), have reported that *p*-aminohippuric acid does not antagonize the bacteriostatic effect of the sulfonamides *in vitro*. However, Meunzen, *et al.* (5), found only N-acetyl *p*-aminobenzoic acid, and no N-acetyl *p*-aminohippuric acid, in the urine of man following ingestion of *p*-aminobenzoic acid; the compound was isolated in the pure crystalline state and its identity proved directly. Cohen, *et al.* (6) showed that under conditions similar to those in which Fraction C is obtained, *p*-aminobenzoic acid is readily extracted, while *p*-aminohippuric acid is extracted to only a very slight extent. Therefore, if the compound found in Fraction C were actually *p*-aminohippuric acid rather than *p*-aminobenzoic acid, it would be necessary that a major proportion of the injected procaine be converted to *p*-aminohippuric acid, contrary to the findings reported here and in many other reports (e.g., refs. 5, 7, 8). Further, Williams (9) points out that there is no evidence that *p*-aminohippuric acid is formed. It is therefore believed that the identities of the components are correctly assigned.

It is evident from the accompanying table that the major portion of the procaine was converted to N-acetyl *p*-aminobenzoic acid. This compound was present in every sample in concentrations from 0.2 γ/ml. to 1.5 γ/ml. N-acetyl procaine was found in 26 of the 30 samples; 22 in concentrations of 0.1 γ/ml. or less and no higher than 0.4 γ/ml. in the remaining four. Free *p*-aminobenzoic acid was found in only 7 of the 36 cases; 6 showing a concentration of 0.1 γ/ml. or less and the remaining one 0.3 γ/ml. The antagonism of N-acetyl procaine or N-acetyl *p*-aminobenzoic acid toward the sulfonamides is negligible, about $^1/_{10,000}$ that of free *p*-aminobenzoic acid (10). Further, it has been shown by Zehender (8) that N-acetyl *p*-aminobenzoic acid is not hydrolyzed to free *p*-aminobenzoic acid *in vivo* or *in vitro* at the *p*H of blood when incubated at 37° for eight days; hence there is no probability of *p*-aminobenzoic acid being regenerated from N-acetyl *p*-aminobenzoic acid. Lawrence, *et al.* (11), showed that N-acetyl procaine lacks antagonistic effect. Zehender (8) also pointed out that the degree and rate of acetylation is dependent upon the amount of acetylation precursor in the blood, and that smaller doses are acetylated to a greater degree than larger doses. Since the usual daily dose of aqueous procaine penicillin is only 300,000 units or one-fifth of the amount used in these experiments, it is likely that the amount of free *p*-aminobenzoic acid would be much smaller and the safety factor greater than would be indicated by the results obtained. It should be noted that

TABLE I.—CONCENTRATION OF PROCAINE DERIVATIVES IN THE SERUM OF PATIENTS RECEIVING 1.5 MILLION UNITS OF AQUEOUS PROCAINE PENICILLIN

Penicillin, Units/ml.	No. of Specimens	Average Concentration in γ/ml. Serum		
		P. A. B. A.	*N*-Acetyl P. A. B. A.	*N*-Acetyl Procaine
0.5	3	0.03	0.33	0.03
1.0	7	0.0	0.30	0.13
2.0	14	0.007	0.55	0.085
4.0	9	0.055	0.60	0.10
8.0	3	0.07	0.90	0.07

there appears to be some correlation between the penicillin blood level and the concentration of N-acetyl *p*-aminobenzoic acid, while this is not seen with *p*-aminobenzoic acid or N-acetyl procaine. The findings indicate, therefore, that procaine penicillin may be safely administered in conjunction with sulfadiazine, sulfathiazole, and sulfapyridine, but may result in the production of concentrations of *p*-aminobenzoic acid which *in vitro* have been found to cause antagonism toward sulfanilamide.

SUMMARY

Three hours after the intramuscular administration of 1.5 million units of procaine penicillin, the greater proportion of the procaine is converted to N-acetyl-*p*-aminobenzoic acid. A very small amount is converted to N-acetyl procaine, while in 7 out of 36 cases very low concentrations of free *p*-aminobenzoic acid were found. These findings indicate that procaine penicillin may be safely administered with sulfadiazine, sulfathiazole, and sulfapyridine, but may be antagonistic toward sulfanilamide.

REFERENCES

(1) Northey, E. H., "The Sulfonamides and Allied Compounds," Reinhold Publishing Corp., New York, 1948, p. 466 *et seq.*
(2) Brodie, B. B., Lief, P. A., and Poet, R., *J. Pharmacol. Exptl. Therap.*, **94**, 359(1948).
(3) Wyss, O., Grubaugh, K. K., and Schmelkes, F. C., *Proc. Soc. Exptl. Biol. Med.*, **49**, 618(1942).
(4) Beyer, K. H., Mattis, P. A., Patch, E. A., and Russo, H. F., *J. Pharmacol. Exptl. Therap.*, **84**, 136(1945).
(5) Meunzen, J. B., Cerecedo, L. R., and Sherwin, C. P., *J. Biol. Chem.*, **67**, 469(1926).
(6) Cohen, P. B., and McGilvery, R. W., *ibid.*, **166**, 261 (1946).
(7) Strauss, E., Lowell, F. D., and Finland, M. J., *Clin. Invest.*, **20**, 189(1941).
(8) Zehender, F., *Helv. Chim. Acta.*, **26**, 1338(1943).
(9) Williams, R. T., "Detoxication Mechanisms," John Wiley and Sons, New York, 1947, p. 107.
(10) Woods, D. D., *Brit. J. Exptl. Path.*, **21**, 74(1940).
(11) Lawrence, C. A., and Goetchius, G. R., *Proc. Soc. Exptl. Biol. Med.*, **57**, 180(1944).

A Study of the Surface Lipids of Skin*

By J. E. PRITCHARD†, L. D. EDWARDS, and J. E. CHRISTIAN

A new method for the quantitative determination of human and animal surface lipids is described. Average lipid values for men and women have been determined and frequency distribution curves are presented. The effects of soaps, astringents, and temperature on the lipid production of human beings have been studied. The action of sex hormones, thyroxin, and thiouracil on the sebaceous gland activity of the albino rat has also been considered.

THE FACTORS which control the rate of secretion of the surface lipids have not been identified conclusively, but several possibilities have been advanced. These include sex hormones (1-3), amount of fats consumed (4-6), environmental temperature (4, 6, 7), and the activity of the thyroid gland (8, 9).

The methods of estimating the surface lipids have for the most part been time-consuming and unreliable. They have included gravimetric (4, 6-8), titrimetric (10, 11), nephelometric (12), and one involving surface tension effects (13). Since these methods appeared to be unsatisfactory either from the standpoint of speed and precision or because of a lack of specificity for surface lipids, a new method was developed. This method has been used to obtain normal lipid values for humans and animals and to study the effect of various factors on the activity of the sebaceous glands.

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EXPERIMENTAL

Human Surface Lipids

Analytical Method.—The area to be sampled is defined by means of a circular wire form which is held against the skin with an elastic band. A filter paper having a diameter of 2.45 cm. is mounted on the sampling apparatus (Fig. 1) and pressed against the skin by the action of the syringe plunger which is activated by a gas pressure of 20 lb. per sq. in. The filter paper is rotated one-quarter turn after each half minute of contact until a total of four contacts and a net contact time of two minutes have been completed. This technique has been shown to remove virtually all the available lipids from the surface of the skin. The filter paper is next washed with 15–20 cc. of ether and this ethereal solution of the lipids is assayed for esters.

The determination of the esters is accomplished spectrophotometrically by a method adapted from that of Hill (14). To the ether solution of the lipids is added 1 cc. of alcoholic sodium hydroxide which is prepared by first dissolving 15 Gm. of sodium hydroxide in 50 cc. of water, pipetting 2 cc. of this solution into a 50-ml. flask, and then filling to the mark

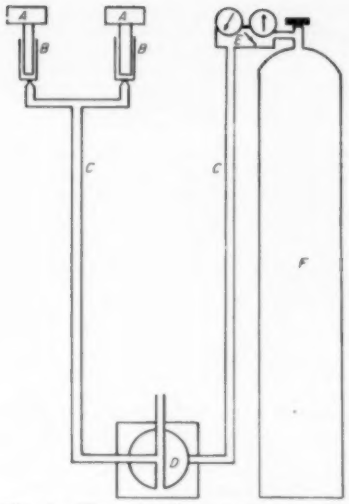

Fig. 1.—The sampling apparatus for humans.

A, Sponge rubber pads which hold filter paper; *B*, 5-cc. syringes; *C*, rubber tubing; *D*, three-way stopcock; *E*, pressure regulator; *F*, cylinder of compressed gas.

with 95% ethanol. One cubic centimeter of hydroxylamine solution which is prepared by dissolving 0.6 Gm. of hydroxylamine hydrochloride in 50 cc. of ethanol is also added. The mixture is then evaporated on a hot plate maintained at a temperature of 160 ± 10°. The sample is removed from the hot plate and cooled in water a few seconds before dryness is reached. After the residue has cooled for two or three minutes, 20 cc. of ferric perchlorate solution is added to produce the red complex. This solution is prepared according to the procedure of Hill (14). The intensity of color which is developed by the sample is compared to that developed from known quantities of cholesteryl stearate; thus, the lipid values reported in this work refer to the cholesteryl stearate equivalent.

The reagents used in the assay procedure were the commercially available U. S. P. or "reagent" grade materials, and none, with the exception of the ether, required any special treatment before use. Most of the available ether contained measurable quantities of esters, but these were readily removed by distilling the ether from alcoholic potassium hydroxide. The cholesteryl stearate which was used as the primary standard was prepared by the method of Cataline, *et al.* (15). Sodium chloride, moisture, oleic acid, and urea in the quantities available from human skin do not interfere.

The over-all speed and precision of the method is indicated by the fact that duplicate determinations on 20 human subjects can be completed in three hours. The average deviation of the two values of each pair from their mean is of the order of ±6%.

Normal Lipid Values.—Lipid values were determined for two adjacent forehead areas of 56 women and 151 men. In each case the forehead area of the subject was wiped free of oil, cosmetics, and the like at 1 p. m. on the day of the test. The subject remained in the laboratory for the next three hours during which time he was instructed to refrain from touching the test area or interfering with the normal replenishment of the lipids in any way. The actual testing was then accomplished in the manner previously described. The sampling interval was set at three hours, since it was found that this was the minimum time which would allow complete recovery of the normal lipid level for all individuals.

The results of this study are recorded in the form of frequency distribution curves (Table I and II, Fig. 2) which utilize 10 cells representing values of 0.0 to 0.5 mg. of lipids per sq. cm. of area. Both the curves, particularly that for women, show a skewness in a positive sense. The over-all range for both men and women is 0.05 to 0.50 mg./cm.^{2}, but the mean for men is 0.19 mg./cm.^{2}, while that for women is 0.17 mg./cm.^{2} The median and the mode for men are 0.18 mg./cm.^{2} and 0.17 mg./cm.^{2}, while for women these values are 0.15 mg./cm.^{2} and 0.11 mg./cm.^{2}, respectively. These differences are not great but nevertheless appear to be real. No relationship between age or color of hair and lipid values could be found.

Effect of Soaps on Surface Lipids.—The forehead areas of 5 individuals were divided into two equal parts for the purpose of the experiment. One area was washed with a cotton swab carrying the soap solution to be tested, rinsed with distilled water, and dried by gently wiping with fresh cotton swabs.

TABLE I.—SUMMARY OF FREQUENCY DISTRIBUTION DATA FOR WOMEN

Cell No.	Lipids Mg./Cm.^{2}	No. of Subjects in Cell	Per Cent of Total
0	0.00–0.02	0	0.0
1	0.03–0.07	4	7.1
2	0.08–0.12	18	32.2
3	0.13–0.17	13	23.2
4	0.18–0.22	10	17.9
5	0.23–0.27	5	8.9
6	0.28–0.32	4	7.1
7	0.33–0.37	0	0.0
8	0.38–0.42	0	0.0
9	0.43–0.47	1	1.8
10	0.48–0.52	1	1.8

TABLE II.—SUMMARY OF FREQUENCY DISTRIBUTION DATA FOR MEN

Cell No.	Lipids Mg./Cm.^{2}	No. of Subjects in Cells	Per Cent of Total
0	0.00–0.02	0	0.0
1	0.03–0.07	2	1.3
2	0.08–0.12	28	18.6
3	0.13–0.17	42	27.8
4	0.18–0.22	43	28.4
5	0.23–0.27	21	13.9
6	0.28–0.32	7	4.6
7	0.33–0.37	5	3.3
8	0.38–0.42	1	0.7
9	0.43–0.47	1	0.7
10	0.48–0.52	1	0.7

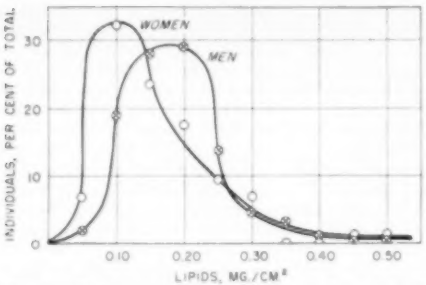

Fig. 2.—Frequency distribution curves for men and women.

This procedure was intended to simulate common cleansing practices. The other forehead area was freed of the accumulated lipids and foreign matter by means of absorbent paper. After a period of two hours the quantity of lipids which had accumulated on the surface of the two areas was determined.

The soap solutions studied were 0.02 *M* solutions of sodium stearate, sodium palmitate, sodium myristate, sodium laurate, and sodium caprate, and a 0.02 *M* solution of sodium stearate containing lanolin to the extent of 10% by weight of the soap. In every case the washed area of skin exhibited virtually the same lipid value after the two-hour recovery as did the unwashed area.

Effect of Astringents on Lipid Production.—The forehead areas of 5 individuals were cleansed with absorbent paper just prior to the application of the astringent solution. The material to be tested was then spread on one-half of the area by means of a cotton swab and allowed to dry. After a period of two hours the lipid levels on both the treated and untreated areas were determined.

The first astringent solution tested, 10% aluminum chloride, did not alter the rate of lipid replenishment to any appreciable extent. Since it seemed possible that this lack of activity might be due to the inability of the aqueous solution to wet the skin surface uniformly, a second solution containing 10% aluminum chloride in a 1% solution of a wetting agent (Aerosol AY) was studied. This material was likewise without appreciable effect on the final lipid values attained.

Effect of Temperature on Lipid Values.—Twenty-four persons who had been tested at various times during the winter months were again examined during relatively warm weather. The prevailing temperatures in the former case were generally in the vicinity of 5°, while in the latter instance it was 29°. Decreases in lipid values were observed in 7 cases, increases were found in 2, and 15 exhibited no significant change. The average of the group was the same for both the warm and cold test periods.

Animal Surface Lipids

Analytical Method.—The surface lipids of animals are determined indirectly by measuring the quantity of esters in a given weight of the animal hair. Approximately 100 mg. of hair is needed for the test and this is obtained by clipping small samples from 8 or 10 scattered areas on the back and sides of the animal. The sample is then weighed to the nearest milligram and immersed in a few cubic centimeters of ether. After this ethereal solution and two subsequent ether washings have been filtered through a sintered glass filter, the filtrate is assayed for esters by the procedure previously described.

Effect of Sex Hormones on the Lipid Values of Albino Rats.—It has been reported that the sebaceous glands of human beings exhibit increased activity at the time of puberty (1, 3), and it is generally assumed that this phenomenon is closely associated with the increase in the supply of hormones which occurs during this period. The 32 albino rats which were utilized in this study were between five and six weeks of age, initially, and were studied over a period of six weeks. The animals were divided into 5 groups for the purposes of the experiment. Group 1 consisted of 5 males which were used as controls. Group 2 included 5 males receiving peanut oil in quantities of 0.2 cc. weekly for the first three weeks and 0.4 cc. twice weekly for the last three weeks. Group 3 contained 9 males receiving 0.5 mg. of testosterone propionate in 0.2 cc. of peanut oil weekly for the first three weeks and 1.0 mg. in 0.4 cc. twice weekly for the final three weeks. Group 4 was composed of 4 females receiving 0.2 cc. of peanut oil weekly for three weeks followed by 0.4 cc. twice weekly for the last three weeks. Group 5 consisted of 9 females receiving 0.05 mg. of theelin in 0.2 cc. of peanut oil weekly for three weeks and 0.1 mg. in 0.4 cc. of oil twice weekly during the final three weeks. All of the dosages of hormones are in the range reported by Griffith and Farris (16) to give typical therapeutic results. They were increased at the end of the first three-week period because preliminary tests indicated that no appreciable changes in the lipid values had occurred.

The average per cent of lipids in the hair initially and at the end of six weeks was as follows for each group: Group 1, 1.0 and 1.2; Group 2, 1.1 and 1.4; Group 3, 1.3 and 1.7; Group 4, 0.8 and 0.9; Group 5, 1.2 and 0.9. All of these changes are relatively small and even the highest final value is considerably below the average of 2.3% which was observed for mature animals. It must be concluded that the administration of peanut oil and hormones to immature albino rats in the manner which has been described produces no significant changes in the lipid content of the animal hair.

Effect of Thyroxin and Thiouracil on Lipid Values of Albino Rats.—The 25 mature animals which were utilized in this study were divided into 3 groups. Group 1 was composed of 5 animals which were used as controls. Group 2 consisted of 10 animals which received 0.1 mg. of thyroxin subcutaneously each day for the first three weeks and 0.2 mg. daily for the last three weeks. Group 3 was composed of 10 animals receiving 0.1 Gm. of thiouracil orally each day for the first three weeks, 0.2 Gm. daily during the fourth week, and 0.1 Gm. daily for the final two weeks. All of these quantities are in a range which has been reported to be therapeutically active (17, 18). The dosages of thyroxin and thiouracil were increased at the end of the first three-week period because preliminary tests indicated that little if any change had occurred in the lipid values during this time. However, the death of two of the animals in

the thiouracil group indicated that the dosage of this drug should be maintained at the lower level.

The average lipid values of both the control and the thyroxin-treated animals remained exactly constant at 2.0% and 2.1%, respectively. The average body weight of the control group increased from 325 Gm. to 355 Gm., while that of the animals receiving thyroxin decreased from 308 Gm., initially, to 288 Gm. at the end of the six-week period. The lipid values of the 8 animals which survived the thiouracil treatment increased from an average of 2.3% to 3.0%, while the average body weight decreased from 299 Gm. to 280 Gm. In view of the fact that both of the other groups of animals exhibited constant lipid values throughout the period of the test, the increase shown by the thiouracil group is believed to be significant.

SUMMARY

1. A method has been developed for the quantitative determination of human and animal surface lipids.

2. The over-all range of forehead lipid values for both men and women was found to be 0.05 to 0.50 mg./cm.^{2}. The mean for men is 0.19 mg./cm.^{2}, while that for women is 0.17 mg./cm.^{2}.

3. The application of soaps and astringents to the skin as well as changes in environmental temperatures were without effect on the lipid values of a limited number of human subjects.

4. Testoterone and theelin administered to immature male and female rats, respectively, produced no appreciable change in the lipid values of the animals.

5. Administration of thyroxin to albino rats produced no change but treatment with thiouracil resulted in significant increases in the lipid values of the animals.

REFERENCES

(1) Rothman, S., *Psychosomat. Med.*, **7**, 90(1945).
(2) Hooker, C. W., and Pfeiffer, C. A., *Endocrinology*, **32**, 69(1943).
(3) Hamilton, J. B., *J. Clin. Endocrinol.*, **1**, 570(1941).
(4) Perutz, A., and Lustig, B., *Biochem. Z.*, **261**, 128 (1933).
(5) Somekawa, E., *Sci. Papers Inst. Phys. Chem. Research (Tokyo)*, **35**, 121(1938).
(6) Kuznitzky, E., *Arch. Dermatol. Syphilis*, **114**, 691 (1913).
(7) Rabbeno, A., *Giornale Italiano Delle Mallattie Veneree E. Della Pella*, **65** (II), 1509(1924).
(8) Jurenka, W., *Z. Züchf. Reihe B. Tierzücht. u. Züchtungsbiol.*, **31**, 373(1935).
(9) Abelin, I., and Klinger, K., *Helv. Physiol. et Pharmacol. Acta*, **6**, 1(1948).
(10) Pachur, R., *Arch. Dermatol. Syphilis*, **162**, 253(1930).
(11) Schur, H., and Goldfarb, L., *Wien. klin. Wochschr.*, **40**, 1255(1927).
(12) Emanuel, S. V., *Acta Dermato-Venereol.*, **17**, 444 (1936).
(13) Peukert, K., *Arch. Gewerbepath. Gewerbehyg.*, **10**, 53 (1940).
(14) Hill, U. T., *Ind. Eng. Chem., Anal. Ed.*, **18**, 317(1946).
(15) Cataline, E. L., *et al.*, THIS JOURNAL, **33**, 107(1944).
(16) Griffith, J. Q., and Farris, E. J., "The Rat in Laboratory Investigation," J. B. Lippincott, Philadelphia, 1942, pp. 308, 319.
(17) De Caro, L., and Giani, M., *Arch. exptl. Path. Pharmakol.*, **173**, 398(1935).
(18) Marine, D., and Baumann, E. J., *Am. J. Physiol.*, **144**, 69(1945).

Observations on the Leaves of Species of Viburnum. II*

By HEBER W. YOUNGKEN

A report is given of further studies on leaves of medicinal Viburnum species. The histology of the leaf of *Viburnum trilobum* is described. The results of additional studies on the vein islet numbers, palisade ratios, and stomatal indices of *Viburnum prunifolium*, *V. rufidulum*, and *V. cassinoides* are given.

IN A PAPER dealing with the leaves of several medicinal species of Viburnum (1), the author described the physical characteristics, the histology, and the vein islet numbers of the leaves of *Viburnum prunifolium*, *V. rufidulum*, and *V. cassinoides* and presented a preliminary report on the palisade ratios and stomatal indices of these. He has extended his studies to the leaves of these and other medicinal Viburnums and, in this paper, presents the results of observations on the leaves of *Viburnum trilobum* Marshall and on the vein islets, palisade ratios, and stomatal indices of *Viburnum lentago* L., *V. prunifolium* L., *V. rufidulum* Raf., *V. cassinoides* L., and *V. trilobum* Marsh.

The leaves of all of these were gathered by the author from plants growing in the Arnold Arboretum of Harvard University and in the medicinal plant garden of the Massachusetts College of Pharmacy at Jamaica Plain, Mass. The methods employed were similar to those discussed in

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the previous paper on this subject by the writer, with the following exceptions: At least five leaves of each species were used in making palisade ratio and vein islet number counts which were recorded for apex, margin, center, and base and averaged, the concept of Benedict (2) was also applied in counting vein islets, and the camera lucida was used in sketching vein islets and palisade cells in vein islet number and palisade ratio determinations.

HISTOLOGY OF THE LEAF OF *VIBURNUM TRILOBUM* Marshall (*VIBURNUM OPULUS* L. VAR. AMERICANUM (MILLER) AITON

The leaf exhibits dorsiventral structure.

Lamina.—The upper epidermis consists of a layer of epidermal cells with sinuate anticlinal walls in the interneural regions and rectilinear anticlinal walls over the veins. It bears numerous unicellular, straight to curved, nonglandular hairs of yellowish color and numerous stalked, capitate and peltate glandular hairs. The basal cell of the stalk of each kind of hair is wedged in between the surrounding cells of the epidermis. An occasional short-stalked, clavate glandular hair was observed arising from this epidermis in the groove of the midrib.

The mesophyll is differentiated into an upper layer of palisade parenchyma and, in the interneural region, of 4 or 5 layers of spongy parenchyma containing chloroplasts and rosette aggregates of calcium oxalate. While arm-palisade cells were found, in the sections examined, they appeared fewer in number as compared with the palisade parenchyma region of the European *Viburnum Opulus*. The palisade parenchyma cells of the midrib region appeared mostly rectangular to quadrangular in transverse sections and typically columnar in the interneural regions of the lamina. Coursing through the spongy parenchyma are fibrovascular bundles which are surrounded by a layer of clear, rounded, border parenchyma cells, as observed in transverse sections.

In the midrib region the mesophyll shows a broad, crescent-shaped meristele (trace) embedded in parenchyma and consisting of numerous narrow collateral bundles separated by medullary rays, the latter mostly 1 cell in width. The xylem region of these bundles showed tracheary tissue with spiral and scalariform markings. Rosette aggregates and crystal fibers containing rosette aggregates of calcium oxalate occur in the parenchyma zone A layer or two of collenchyma cells occur beneath each epidermis of the midrib region.

The lower epidermis in cross section consists of a layer of epidermal cells of rectangular or angularly rounded form and numerous interspersed elliptic stomata. In the region of the veins and midrib the outer walls of the epidermal cells are extended as short papillae. Nonglandular and glandular hairs of similar types to those found in the upper epidermis occur even more numerously on this epidermis. In surface sections the lower epidermal cells show deeply undulate anticlinal walls, the crests of the bulges being extended in linear fashion. The nonglandular hairs of both epidermises occur as single outgrowths of straight, curved, or appressed character and also as double and tufted outgrowths. They show a nearly keystone-shaped basal portion with a beaded wall and are wedged in between a circle of surrounding epidermal cells which, as frequently noted in the vein regions, are elevated above the level of the other epidermal cells. These hairs measure up to 570 μ in length. They possess a relatively thin wall and a broad lumen extending to the pointed tip. The basal region of the double hairs shows pits in the common communicating wall. The stomata were up to 42 μ in length. There was no regularity in the arrangement of the subsidiary cells around the guard cells of the stomata as noted

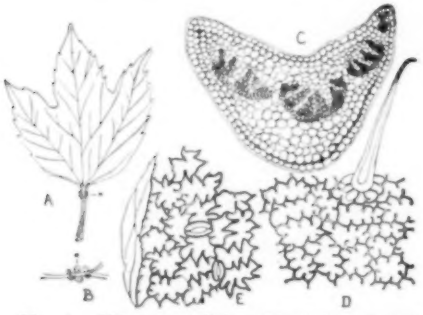

Fig. 1.—*Viburnum trilobum* Marshall. *A*, leaf, showing palmately 3-lobed lamina, and petiole with groove extending its entire length, bearing extrafloral nectaries (*N*) in its upper portion (× 1/3); *B*, enlarged view of six nectaries (*N*) as viewed with a hand lens (× 1/2); *C*, basal region of petiole, as seen in a magnified cross section which was cut slightly above the point of attachment of the petiole to the stem, and showing 3 large traces and a small branch trace to the right of the right lateral trace; *D*, upper epidermis of leaf blade bearing a unicellular, nonglandular hair; *E*, lower epidermis of leaf blade. *C*, *D*, *E*, magnified.

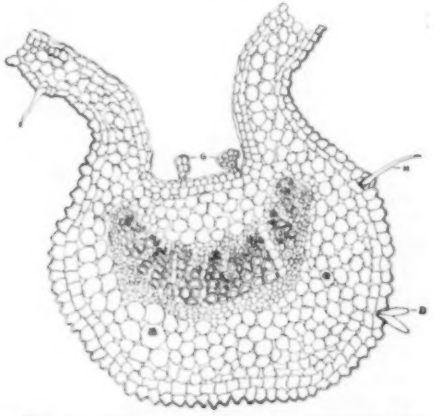

Fig. 2.—Transverse section of a representative portion of the lamina of *Viburnum trilobum* showing the midrib and adjacent interneural regions. Magnified. The crescent-shaped trace (meristele) is shown in the center of the midrib. *H*, deep-seated, nonglandular hair; *D*, double hair; *G*, glandular hairs.

by Solereder (3) and others for other species of the *Caprifoliaceæ*. The peltate hairs possessed a shield of from 4 to 8 cells.

Petiole.—The petiole of *Viburnum trilobum* is grooved on its ventral surface throughout its entire length. In its basal portion it bears a pair of lateral, filiform stipules and in its upper region from 3 to 8 extrafloral nectaries which arise ventrad from its wings. Transverse sections of it are concavo-convex in outline and exhibit the following histological structures:

Serial transverse sections through the basal region of the petiole show a cutinized epidermis consisting of irregularly rounded, comcal, and tangentially elongated epidermal cells. Beneath the epidermis there occurs a zone of collenchyma of from 1 to 6 layers of collenchyma cells and, following it, a broad area of parenchyma, scattered cells of which contain rosette aggregate crystals of calcium oxalate up to 38 μ in diameter. Embedded in the central parenchymatous zone at the very base of the petiole are three leaf traces of reniform to crescentic outline. Sections cut above the base showed, in addition, one or two vascular branch traces, arranged on either side of the larger lateral traces. The traces (meristeles) consist of radially arranged collateral bundles separated by rays, the latter mostly 1 cell wide.

Longitudinal sections of the petiole showed the parenchyma cells to be axially elongated and to possess beaded and pitted walls. Both scalariform and spiral tracheids occurred in the xylem, and long rows of cells, each containing a rosette aggregate crystal of calcium oxalate (crystal fibers) occurred in the parenchyma adjacent to the traces.

Serial transverse sections of the upper region of the petiole frequently have their outer epidermal cell walls extended as papillae, and these walls are covered by a prominent cuticle. Glandular hairs with globular and club-shaped heads and with basal part of stalk embedded between adjacent epidermal cells as well as peltate hairs are especially prominent on the upper epidermis in the groove region.

Transverse sections of the nectary region of the petiole show spiral tracheids issuing from a lateral trace and extending to a mass of secretory cells which border the cavity of the nectary.

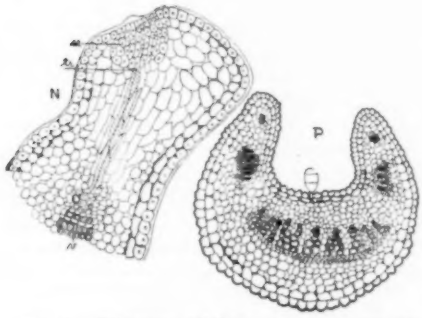

Fig. 3.—*Viburnum trilobum*. *P*, transverse section of the petiole near its summit. Note the deep groove on its upper surface flanked on either side by a wing, the three original traces which entered the base of the petiole, and the two small branch traces in the distal portion of the wings. A deep-seated clavate hair is seen arising from the upper epidermis in the bottom of the groove. *N*, transverse section of the end of the petiolar wing including the extra floral nectary. Note the trace at the lower end with tracheary tissue (*v*); the tracheids (*tr*) extending from the trace to a mass of secretory cells which secrete nectar into the nectar cavity. Magnified.

PALISADE RATIOS OF VIBURNUM LEAVES

The average palisade ratios for the base, margin, center, and apex of 5 different Viburnum leaves were determined by the following method: Pieces cut from these areas were cleared by boiling them in chloral hydrate solution (50 Gm. chloral hydrate and 20 cc. distilled water) and mounted in a drop of chloral solution and a drop of diluted glycerin. The camera lucida and 4 mm. objective were employed in the examination. The outlines of the upper epidermal cells were drawn in ink on a sheet of paper. The palisade cells beneath each 4 adjacent epidermal cells were counted, together with any palisade cells more than half within the area of the epidermal cell, and these were traced within the inked outlines of epidermal cells with a pencil. (See Fig. 4.) For each of the Viburnum leaves, counts of the palisade cells were made in 5 different fields of the piece of each region, the total number for each region separately added and divided by 20 (4 × 5). The average palisade ratio for the leaf was computed by adding the figures representing the average palisade ratios of the regions and dividing by 4.

The results were as follows:

Viburnum Cassinoides Palisade Ratios.—Base, 2.7. Margin, 2.75. Center, 2.65. Apex, 2.65. Average, 2.68.

Viburnum Prunifolium Palisade Ratios.—Base, 4.5. Margin-center, 4.7. Center, 4. Apex, 4.5. Average, 4.42.

Viburnum Lentago Palisade Ratios (2 series counted).—

(1) Base, 2.4. Margin, 2.35. Center, 2.3. Apex, 2. Average, 2.26.

(2) Base, 3.25. Margin, 4. Center, 3.5. Apex, 3.5. Average, 3.56.

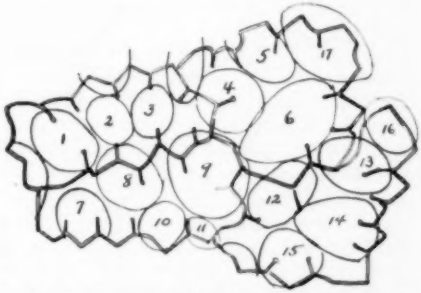

Fig. 4.—Camera lucida sketch of four adjacent upper epidermal cells and subjacent palisade parenchyma cells in a cleared strip of leaf of *Viburnum trilobum*. Those palisade cells completely within the area of each epidermal cell and those more than half within the area were numbered and the total within the four cell areas divided by 4 to determine the palisade ratio. The palisade ratio in this illustration is 4.25.

Viburnum Rufidulum Palisade Ratios.—Base, 2.5. Margin, 2.35. Center, 2.35. Apex, 2.3. Average, 2.31.

Viburnum Trilobum Palisade Ratios.—Base, 4.27. Margin-center, 8. Center, 6.23. Apex, 4.40. Average, 5.75. For the margin-center of this leaf the palisade ratio range was from 6 to 11.

STOMATAL INDEX OF VIBURNUM LEAVES

The stomatal index determinations were made by the same method reported in the previous paper of the author. The results were as follows: *Viburnum cassinoides*, 10.3; *Viburnum lentago*, 8.4; *Viburnum prunifolium*, 8.46; *Viburnum rufidulum*, 10.6; *Viburnum trilobum*, Apex, 9.28; Margin-center, 13.5; Base, 13.6.

VEIN ISLET NUMBER DETERMINATIONS

The vein islet number determinations were made by the following method: Pieces about 6 mm. square were cut from the apex, margin, center, and base of each of the leaves of 5 different species of Viburnum, placed in a small beaker and boiled in a strong solution of chloral hydrate until the tissues were cleared, transferred to a slide, and mounted in diluted glycerin solution. By means of a stage micrometer placed on the stage of the microscope which carried a 16-mm. objective and an attached camera lucida, a line equivalent to 1 mm. as seen through the microscope was drawn on paper and a square constructed therefrom. The slide carrying the mount of the cleared leaf segment was placed on the stage, focused, the paper moved so that the square was seen in the ocular, and the veins included in the square drawn and the vein islets numbered. The islets overlapping two adjacent sides of the square millimeter were counted along with the islets surrounded by veins.

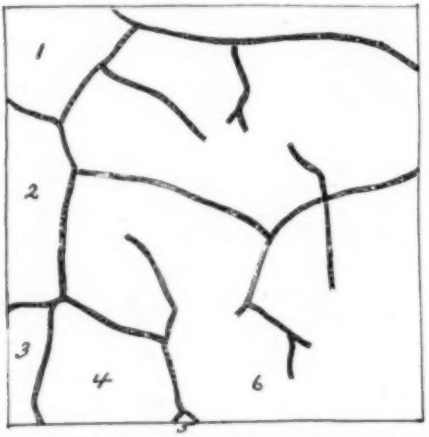

Fig. 5.—Camera lucida drawing of the veins within a ruled 1-mm. square area in a cleared strip cut from the base region of a leaf of *Viburnum trilobum*. Magnified. The numbered areas are those counted as vein islets, following the rule of counting areas of mesophyll completely surrounded by veins and those overlapping partial islets on two sides of the square millimeter which are adjacent.

In the case of *Viburnum trilobum* leaf (Table I), a 4-square-mm. area was counted for each of four regions of 5 leaves and the number divided by 4 to obtain the average vein islet number for 1 mm. The vein islet number determinations for *V. lentago* leaf and *V. cassinoides* leaf are given in Tables II and III.

TABLE I.—VIBURNUM TRILOBUM LEAF[a]

Leaf No.	1	2	3	4	5	Average Vein Islet No.
Apex	6.5	7.75	7.25	9	7	7.5
Margin	4.5	5	4	5.5	5.5	5
Center	5.25	6.5	4.75	6.5	6.5	5.9
Base	5	5.5	5.5	6	8.25	6

[a] Average vein islet number for 5 leaves of *Viburnum trilobum* by the Benedict method is 6.

TABLE II.—VIBURNUM LENTAGO LEAF[a,b]

Leaf No.	1	2	3	4	5	Average Vein Islet No.
Apex	2	2.5	3	2.5	2.5	2.5
Margin	3.5	3.5	3	3	2.5	3.1
Center	4.5	4	4.5	4.5	4.5	4.5
Base	2.5	2.5	3	2.5	2	2.5

[a] Average vein islet number for 5 leaves of *Viburnum lentago* is 3.15.
[b] By method of counting completely enclosed and more than half enclosed areas.

TABLE III.—VIBURNUM CASSINOIDES LEAF[a,b]

Leaf No.	1	2	3	4	5	Average Vein Islet No.
Apex	11	9	10	11	10	10.2
Margin	9	8	8	9	10	8.8
Center	10	9	10	9	9	9.5
Base	10	11	10	9	10	10

[a] The average vein islet number for 5 leaves of *Viburnum cassinoides* by method *b* was 9.6. The average vein islet number by the method of counting only areas of mesophyll completely surrounded by veins in 5 fields of apex, base, and margin-center was 3.77. (Benedict method).
[b] By method of counting completely enclosed and more than half enclosed areas.

Determinations of the vein islet numbers on 5 leaves of *V. prunifolium* gave the following mean averages in four regions, viz.: Apex, 5.8; Margin, 3.6; Center, 4.6; Base, 4.2; General average, 4.6.

Determinations of the vein islets of 5 additional leaves of *V. rufidulum* gave the following mean averages in four regions, viz.: Apex, 3.7; Margin, 3.7; Center, 3.5; Base, 3.1; General Average, 3.5.

SUMMARY

1. The histology of the leaf of *Viburnum trilobum* is described and illustrated by drawings.

2. Average vein islet number values, palisade ratios, and stomatal indices are reported for leaves of *V. lentago*, *V. trilobum*, *V. cassinoides*, *V. prunifolium*, and *V. rufidulum*.

3. The average vein islet number for 5 leaves of *V. lentago* was found to be 3.15; for *V. trilobum*,

6; for *V. cassinoides*, 3.77 (by method of counting mesophyll areas completely enclosed by veins) and 9.6 by method of counting completely enclosed and more than half enclosed areas of the mesophyll; for *V. prunifolium*, 4.6; for *V. rufidulum*, 3.5.

4. The average palisade ratio for *V. lentago* was 2.91 with an average range of 2 to 4.

The average palisade ratio for *V. trilobum* was 5.75 with an average range of 4.27 to 8; for *V. cassinoides*, 2.68 with an average range of 2.65 to 2.75; for *V. prunifolium*, 4.42 with an average range of 4 to 4.7; for *V. rufidulum*, 2.31 with an average range of 2.25 to 2.35.

5. The stomatal indices for leaves of *V. lentago* and *V. trilobum* are reported for the first time and the results of additional stomatal index determinations on more materials given for *V. cassinoides*, *V. prunifolium*, and *V. rufidulum*.

REFERENCES

(1) Youngken, H. W., THIS JOURNAL, **37**, 349(1948).
(2) Benedict, H. M., *Memoirs N. Y. Exper. Agr. Sta.* 281, 1915.
(3) Solereder, H., "Systematic Anatomy of the Dicotyledons," rev. by D. H. Scott, Clarendon Press, Oxford, Vol. 1 1908, p. 441.

Some New Soluble Forms of Theophylline*

By J. M. HOLBERT, I. W. GROTE, and J. W. LEMAISTRE

Investigations relating to theophylline-sodium glycinate have been extended to cover the behavior of the sodium and potassium salts of glutamic acid, anthranilic acid, and *p*-aminobenzoic acid with theophylline. Caffeine and theobromine are not similarly solubilized. Some physical and chemical properties of the compounds are discussed.

ALTHOUGH theophylline, 1,3-dimethylxanthine, enjoys wide medical acceptance, its usefulness, *per se*, is limited by its relative insolubility in water. When theophylline is combined with such compounds as diethanolamine, sodium acetate, sodium salicylate, and ethylenediamine, the resulting complexes or double salts exhibit a substantial increase in solubility. Unfortunately, the ingestion of these compounds is often associated with more or less gastric distress and nausea. This malaise is thought to be due to the fact that hydrochloric acid in the stomach readily decomposes the double salt into its original components which irritate the gastric mucosa.

In 1947, Krantz, *et al.* (1), reported the preparation of theophylline-sodium glycinate, which was soluble in water to the extent of approximately 1 Gm. in 5 cc. of solution, quite stable to carbon dioxide of the air, and well tolerated in large doses. The structure of theophylline-sodium glycinate is thought to be best represented by the following equilibrium:

$$C_7H_7O_2N_4Na + NH_2CH_2COOH \rightleftharpoons C_7H_8O_2N_4 + NH_2CH_2COONa$$

In an attempt to extend this principle to solubilizing theobromine and caffeine, it was found that the addition of sodium glycinate to hot solutions of theobromine and/or caffeine resulted in the almost quantitative precipitation of these alkaloids.

Recently we have successfully solubilized theophylline by combining it with the alkali metal salts of amino acids other than aminoacetic acid; namely, *p*-aminobenzoic acid, anthranilic acid (*o*-aminobenzoic acid), and glutamic acid. These mixtures are all characterized by ready solubility in water, the potassium salt in each case being more soluble than the corresponding sodium salt.

The combination, theophylline-potassium-*p*-aminobenzoate, exhibited the most striking solubility in water, 53 Gm. of this material being soluble in 100 cc. solution at 28°. It is interesting to note that when equimolar portions of theophylline and *p*-aminobenzoic acid are combined, maximal solubility is reached when the potassium hydroxide used is approximately 69 per cent of that required to combine with both the theophylline and the P. A. B. A. The solubility decreases with further addition of alkali. Table I gives the solubility of equimolar mixtures of theophylline-*p*-aminobenzoic acid with varying amounts of potassium hydroxide.

* Received April 30, 1949, from the Brayten Pharmaceutical Co., Chattanooga, Tenn.
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949

TABLE I.—COMPARISON OF SOLUBILITY OF EQUIMOLAR MIXTURES OF THEOPHYLLINE AND *p*-AMINOBENZOIC ACID WITH VARYING AMOUNTS OF POTASSIUM HYDROXIDE

Mole P. A. B. A.	Mole Theophylline	Mole KOH	Theoretical KOH, %	Solubility—Gm. of Anhydrous Theophylline per 100 Cc. Solution at 28°	Saturated Solution, *p*H
1	1	0.85	42.5	Very low	...
1	1	1.06	53.0	Very low[a]	8.17
1	1	1.25	62.5	Below 10	9.05
1	1	1.38	69.0	25	9.35
1	1	1.50	75.0	24	9.50
1	1	1.70	85.0	18	9.91

[a] The undissolved phase was found by analysis to be practically all theophylline.

TABLE II.—SOLUBILITY OF ALKALI METAL SALTS OF THEOPHYLLINE WITH VARIOUS AMINO ACIDS

Combination	Solubility, Gm. per 100 Cc. of Solution at 28°	Solubility, Gm. of Anhydrous Theophylline per 100 Cc. Solution at 28°	Saturated Solution, *p*H
Theophylline-*p*-aminobenzoic acid:			
Potassium salt	53	25	9.4
Sodium salt	17	8	9.1
Theophylline-anthranilic acid, potassium salt	48	23	9.2
Theophylline-glutamic acid:			
Potassium salt	24	10	9.3
Sodium salt	12	5	9.0
Theophylline-aminoacetic acid:			
Potassium salt	22	10	9.1
Sodium salt	17	8	8.9

A similar mixture of 1 mole theophylline and 1 mole anthranilic acid reaches a maximum solubility with 1.38 mole (69 per cent of the theoretical amount) of KOH.

Table II summarizes the solubility data when theophylline is combined with the alkali metal salts of *p*-aminobenzoic acid, anthranilic acid, aminoacetic acid, and glutamic acid. All of the solutions are reasonably stable to carbon dioxide of the air and as noted in Table II, the *p*H of each saturated solution is acceptable for oral medicinal preparations.

Although animal tests have not been completed on all the compounds, acute toxicity studies on theophylline-potassium *p*-aminobenzoate and theophylline-potassium anthranilate show that the toxicity is equal to that of the theophylline present in each case.

EXPERIMENTAL

The Preparation of Theophylline-Potassium-*p*-Aminobenzoate.—Ninety-one grams (1.38 mole) of potassium hydroxide U. S. P. was dissolved in 1 L. of water, and 180 Gm. (1 mole) of anhydrous theophylline added with stirring. To this mixture was added slowly 137 Gm. (1 mole) of *p*-aminobenzoic acid. Stirring and slight warming effected complete solution. The solution was evaporated *in vacuo* at a temperature of below 50°. The solid material was dried at 85°. Ten cubic centimeters of an aqueous saturated solution of the dried powder at 26° contained 5.26 Gm. of solid.

The Preparation of Theophylline-Sodium-*p*-Aminobenzoate.—To 150 cc. of water containing 6.24 Gm. (0.156 mole) of sodium hydroxide was added 18.0 Gm. (0.1 mole) of theophylline and 13.7 Gm. (0.1 mole) purified *p*-aminobenzoic acid. The solution was filtered to remove a trace of suspended matter, and the filtrate evaporated to dryness at reduced temperature and pressure. The remaining materials in Table II were prepared by identical procedures, with the exception that the ratio of base used varied somewhat to produce maximum solubility.

SUMMARY

1. Six new water-soluble theophylline combinations have been prepared. They are the sodium and potassium salts of theophylline-*p*-aminobenzoic acid and theophylline-glutamic acid, and the potassium salts of theophylline-anthranilic acid and theophylline-aminoacetic acid.

2. The solubilities of these combinations are reported; theophylline - potassium - *p* - aminobenzoate exhibiting a maximum solubility equivalent to 25 Gm. anhydrous theophylline per 100 cc. of solution.

3. Acute toxicity studies on theophylline-potassium-*p*-aminobenzoate and theophylline-potassium anthranilate show that their toxicity is directly related to their theophylline content.

REFERENCE

(1) Krantz, J. C., Holbert, J. M., Iwamoto, H. K., and Carr, C. J., THIS JOURNAL, 36, 248(1947).

Some Esters of Cumic Acid*,†

By E. D. CARKHUFF and L. G. GRAMLING

The cumic esters: methyl, ethyl, propyl, isopropyl, *n*-amyl, *n*-hexyl, 2-ethyl butyl (from 2-ethyl-butanol-1), *b*-phenyl, menthyl, thymyl, *b*-naphthyl, and picrate, were prepared from cuminoyl chloride and the alchol or phenol in question. Their constants are reported. The cuminoyl chloride was prepared by treating cumic acid with thionyl chloride. The action of nitrosyl chloride on cumic acid in the presence of ethanol is discussed. A color reaction based on the formation of ferric cumate is discussed.

CUMIC ACID, or *p*-isopropyl benzoic acid, is a homolog of benzoic acid and possesses many of the reaction characteristics of the latter. In general the formation of esters from cumic acid is the same as from benzoic acid. However, if substitution of various groups on the nucleus is sought, the presence of the isopropyl group must be borne in mind.

Although Richard Meyer (1), in 1883, first synthesized cumic acid, it remained a laboratory chemical until R. C. Palmer (2, 3), of Newport Industries, Inc., Pensacola, Fla., developed, in 1942, a method of synthesizing it from *p*-cymene on a commercial scale.

Due to the fact that cumic acid is a homolog of benzoic acid, it was thought that perhaps some of its esters could be made, having commercial possibilities in the field of odor or flavor. It was with this possibility and intention that this study of cumic acid was undertaken.

EXPERIMENTAL

Only the technical grade of cumic acid[1] was available, hence purification was essential. It was found that crystallization from methanol or ethanol gave the most satisfactory results.

The esters of cumic acid which were made in this investigation were prepared in general from the acid chloride and the alcohols by the Schotten-Baumann reaction.

The acid chloride, cuminoyl chloride, was prepared in two ways: (1) by the action of phosphorus pentachloride, and (2), by the action of thionyl chloride on dry acid. Both methods gave satisfactory results and yields (about 80%). However, thionyl chloride is preferred, due to the fact that the by-products of the reaction, hydrochloric acid and sulfur dioxide, are released as gases and the excess thionyl chloride may be easily distilled off under reduced pressure on a steam bath, leaving reasonably pure cuminoyl chloride.

It was found that cuminoyl chloride is rather quickly hydrolyzed in the presence of moisture or on standing in a closed container; thus the making of large amounts is somewhat impractical.

In order to become more familiar with the ester formation of cumic acid, the methyl, ethyl, propyl, and isopropyl cumate were prepared, purified, and assayed by the general method which follows later. The physical constants of these esters were found to be in close agreement with those given by Bert (4).

The general method of preparation of esters is as follows: one-tenth mole quantities of both cuminoyl chloride and the alcohol were reacted in a suitable container using heat. As the evolution of hydrochloric acid became evident, the source of heat was removed and the reaction allowed to progress spontaneously. However, in some cases it was necessary to reflux in order to carry the reaction to completion. This was especially true in the preparation of the aromatic cumic esters.

It was necessary, in the preparation of the aromatic cumic esters, to employ an inert solvent in order that the reaction and refluxing could be carried out smoothly.

When the liberation of hydrochloric acid had ceased and ester formation was completed, the flask was cooled and dilute potassium hydroxide was added to the crude ester to neutralize the dissolved hydrochloric acid and remove any unreacted cuminoyl chloride as water-soluble potassium cumate.

The ester was then separated from the water and dried with anhydrous sodium sulfate for twenty-four hours. It was then purified by distillation using vacuum when necessary, as with the high boiling esters.

Proof of ester formation was obtained by saponification and identification of the component parts. Saponification of the esters was carried out using 0.2–0.4 Gm. samples which were refluxed with alcoholic potassium hydroxide and titrated with hydrochloric acid using phenolphthalein as the indicator.

After saponification and resulting titration the alcohol was evaporated, the solution made acid, the precipitated cumic acid was identified by melting point and color reaction herein described, and, if possible, the alcoholic portion was recovered and identified.

Table I gives the physical constants and saponification values for the esters prepared in this investigation.

Action of Nitrosyl Chloride on Cumic Acid.—It was thought that by the use of nitrosyl chloride a nitroso group could be introduced into the cumic acid molecule and this subsequently hydrolyzed to the corresponding ketone. However, this does not seem to be the course of the reaction. Ester forma-

* Received April 30, 1949, from the College of Pharmacy, Gainesville, Fla.,
Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

† This paper was abstracted from a thesis presented to the Graduate Council of the University of Florida by E. D. Carkhuff in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

[1] The authors are indebted to Newport Industries, Inc., for the supply of cumic acid used in this investigation.

TABLE I.—DATA FOR CUMIC ESTERS[a]

Ester	Amount in mole, Cuminoyl Chloride	Amount in mole, Alcohol or Phenol	Solvent	Reflux Time, Min.	Yield, %	Boiling Point	Melting Point	D_4^{25}	N^{25}	Saponification No. Found	Saponification No. Calculated	Odor
n-Amyl	0.14	0.11	None	None	76	300.5–302	...	0.9622	1.4970	239.7	239.0	Faint
n-Hexyl	0.10	0.10	None	None	62	311–314	...	0.9757	1.4951	223.3	225.1	Faint
2-Ethyl-butyl	0.10	0.10	None	20	62	310–312	...	0.9585	1.4963	226.1	225.1	Faint
β-Phenylethyl	0.10	0.10	None	None	69	176–177[b]	...	1.0523	1.5505	216.3	209.1	None
Menthyl	0.11	0.11	Xylene	35	54	175–176[c]	...	0.9551	1.5085	184.8	185.8	None
Thymyl	0.10	0.10	Xylene	120	55	174–175[d]	...	1.0257	1.5470	186.9	189.4	None
β-Naphthyl	0.025	0.026	Xylene	120	93	...	100–107	...	...	199.1	199.9	None
Picrate	0.025	0.025	Xylene	60	70	...	184–185	...	...	*	...	None

[a] For data on methyl, ethyl, propyl, and isopropyl esters see Bert (4).
[b] At 0.9 mm.
[c] At 1.0 mm.
[d] At 1.0 mm.
* Since saponification determination was unsatisfactory nitrogen was determined by Kjeldahl method. Calculated *N* was 9.4%, found *N* was 9.2%.

tion seems to result by the method used. This is as follows: 6 Gm. of cumic acid were dissolved in 40 cc. of carbon tetrachloride, placed in a Pyrex flask, and chilled slightly. Five cubic centimeters of ethyl nitrite were then added and dry HCl introduced. The formation of nitrosyl chloride was instantaneous. The flask was then placed in the sunlight and more ethyl nitrite and HCl added as the need arose. This was continued for four hours.

The solution developed into two layers and upon examination of the lower layer, after purification by distillation, the results indicated ethyl cumate.

The explanation offered for ester formation rather than substitution of a nitroso group is that cumic acid readily undergoes esterification and that the ethyl alcohol which existed as a by-product in the formation of nitrosyl chloride supplied the necessary alcohol. Also due to the mild conditions under which the reaction was attempted, substitution on the ring did not occur.

Color Reaction for Cumic Acid.—A color reaction for cumic acid, based on the formation of what is believed to be ferric cumate, was developed for this investigation. The method is as follows: to a small quantity (50 mg. or less) add 2 cc. of distilled water and 1 or 2 drops of concentrated ammonium hydroxide. Boil gently over a free flame to expel the excess ammonia, adding water to replace that lost by evaporation. Continue to boil until the solution is neutral to litmus or until the odor of ammonia is no longer present. Then add 1 drop of freshly prepared neutral ferric chloride solution. The formation of a flesh-colored precipitate constitutes a positive test.

SUMMARY

The cumic esters: methyl, ethyl, propyl, isopropyl, *n*-amyl, *n*-hexyl, 2-ethyl butyl (from 2-ethyl-butanol-1), β-phenyl, menthyl, thymyl, β-naphthyl, and picrate, were prepared from cuminoyl chloride and the alcohol or phenol in question. Their constants are reported. The cuminoyl chloride was prepared by treating cumic acid with thionyl chloride.

The methyl, ethyl, propyl, and isopropyl cumates have pleasant odors; the *n*-amyl, *n*-hexyl, 2-ethyl-butyl, and phenylethyl cumates have faint odors, while the picrate, menthyl, thymyl, and naphthyl cumate are odorless.

The above esters are clear, colorless liquids, with the exception of the picrate and naphthyl cumate which are yellow and flesh-colored solids, respectively.

The action of nitrosyl chloride on cumic acid in the presence of ethanol is briefly discussed.

A color reaction for cumic acid based on the formation of ferric cumate is discussed.

REFERENCES

(1) Meyer, R., *Ann.*, **219**, 243(1883).
(2) Palmer, R. C., *Ind. Eng. Chem.*, **34**, 1028(1942).
(3) Palmer, R. C., and Bibb, C. H. (to Newport Industries, Inc.), U. S. 2,302,462, Nov. 17, 1942, through Chemical Abstracts, **37**, 2390(1943).
(4) Bert, L. M., *Bull. soc. chim. France*, **37**, 1252(1925).

Assays of Cinchona and Nux Vomica by Microsublimation*

By J. A. ZAPOTOCKY† and LOYD E. HARRIS‡

A method of isolating the alkaloids of cinchona and nux vomica is presented. The alkaloids are removed, by microsublimation, from the powdered crude drug which has been treated with sodium carbonate. The procedure can be carried out quantitatively. When the results are compared with those obtained by official assay procedures, the microsublimation method gives results that more accurately represent the alkaloidal content.

THE METHODS of assay for the alkaloidal content of drugs, as illustrated in the monographs found in the United States Pharmacopœia and National Formulary, require an appreciable length of time as well as comparatively large quantities of materials. It is well known that some alkaloids will sublime and that sublimation is a process which may yield purified substances. Since sublimation has been successfully used to separate alkaloids that occur uncombined in plants, it was thought that a modified technique could be devised to separate alkaloids from plants whenever they exist as alkaloidal salts. If the process could be made to proceed quantitatively, a convenient and satisfactory assay method, which seems to offer advantages over standard extraction procedures, would be available.

The fractional separation of the constituents of a mixture of substances (1) and the quantitative recovery of certain alkaloids and nonalkaloids from artificially made samples and tablets (2, 3) has been shown to be possible by means of sublimation. The sublimation temperature of the active constituent had to be below the sublimation or decomposition temperature of the diluents, binders, or lubricants used.

A modification of the glass tube or capillary tube method of sublimation previously used by Ray and Datta (4), Pregl (5), and Benedetti-Pichler (6) was used in this study.

EXPERIMENTAL

The alkaloids present in most plants are in the form of a salt. When the plant is heated without preliminary treatment no crystalline sublimate or only a very minute amount is obtained. In order to determine the effect of an alkaline agent such as sodium carbonate on alkaloidal salts and alkaloids the following experiment was performed:

A series of alkaloids were sublimed at 5 mm. pressure ± 1 mm. and the temperatures of sublimation noted. The same alkaloids were then subjected to the treatment described below.

A small quantity of alkaloid was wetted with enough saturated solution of sodium carbonate to make a paste. The mixture was then evaporated to dryness. The dried mass was reduced to a fine powder and approximately 25 mg. was placed in a tube (17 cm. long and 7 mm. in diameter) sealed at one end. After the powder was placed in the closed end of the tube, the unfilled portion was cleaned with a pipe cleaner. A small pledget of cotton was then placed over the powder. The tube was connected by means of pressure tubing to a Hyvac pump and the pressure reduced to 5 mm. ± 1 mm. Heat was applied, by means of a heated aluminum drying block, to the part of the tube holding the sample, and the sublimation temperature noted.

A group of alkaloidal salts was also subjected to the above treatment. The results are given in Table I.

TABLE I.—LIST OF ALKALOIDS AND ALKALOIDAL SALTS WITH THEIR SUBLIMATION TEMPERATURES BEFORE AND AFTER TREATMENT WITH SODIUM CARBONATE

Substance	Sublimation Temperature, °	Sublimation Temperature after Treatment with Sodium Carbonate, °
Cinchonidine sulfate	...	145
Cinchonine	155	155
Cinchonine sulfate	...	155
Quinidine sulfate	...	145
Quinine	140	140
Quinine sulfate	...	140
Strychnine	165	165
Strychnine sulfate	...	165
Strychnine nitrate	...	165
Strychnine citrate	...	165

The sublimates obtained from the alkaloids were compared microscopically with the sublimates obtained from the respective alkaloids and alkaloidal salts treated with sodium carbonate. In each instance the crystals were found to be identical in shape. The sublimates obtained in these experi-

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An abstract from a part of a dissertation submitted to the Graduate School by J. A. Zapotocky in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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ments were used for future comparisons with the sublimates obtained from plants containing the respective alkaloids.

The Isolation of the Alkaloids of Cinchona and Nux Vomica by Microsublimation.—The dried plant material was reduced to a No. 60 powder and thoroughly mixed in a mortar with sufficient saturated aqueous solution of sodium carbonate to render the drug distinctly wet. This mixture was then permitted to dry at room temperature with periodical trituration to insure uniformity. The dried product was again reduced to a No. 60 powder and approximately 50 mg. were placed in a glass tube (17 cm. long and 7 mm. in diameter) sealed at the end holding the sample. The sample was then heated in an aluminum drying block, under reduced pressure, as described above. The temperature was gradually raised until crystals appeared on the walls of the tube just outside the heated area. The temperature at which sublimation took place was determined by removing the tube from the block periodically, without changing the pressure, and examining for crystal formation under a microscope.

Once crystals began to appear the temperature was kept constant and the shape of the crystals noted. The position of the tube was gradually changed so that a new point was available for collecting the sublimate. When no further sublimation was observed, the temperature was gradually raised. If any new crystals appeared, the temperature at which this occurred was noted.

In order to obtain a fractional separation of the alkaloids, the tube was broken at a point between the sample and the sublimate. The remaining sample was placed in a new tube and the heating process continued at a higher temperature.

The results of the sodium carbonate treatment of cinchona and nux vomica are given in Table II.

closed end of the sublimation tube. This sample was weighed on a microbalance with another tube used as a tare. A piece of cotton was inserted over the sample, the tube was attached to a Hyvac pump, and the pressure was reduced to 5 mm. The end of the tube containing the sample was heated in an aluminum drying block at a temperature range of 140–175°. This was the sublimation temperature range for the four most abundant alkaloids present; namely, quinine, quinidine, cinchonine, and cinchonidine. Heat was applied until crystals stopped forming.

The most rapid method of obtaining complete sublimation was to heat the sample in a tube for successive periods of one hour each. After each heating period, the tube was removed from the heating block and broken at a point between the sample and the sublimate. The sample was transferred quantitatively to another tube, and the sublimate was washed from the tube with alcohol. The process was repeated until microscopic examination revealed no further formation of crystals. The combined alcohol solutions were evaporated in a tared vessel and dried to constant weight at 100–110°. The residue was weighed on a microbalance.

A series of assays was carried out on red cinchona by the above procedure. The same drug was assayed by the N. F. VIII method (7). Table III shows the results of both assays.

Purification of the Alkaloidal Residue Obtained in the N. F. VIII Procedure.—The residues from a series of N. F. assays of cinchona were combined and examined. It was found that one purification of the alkaloids from the chloroform-ether mixture o acid and then to chloroform was insufficient to give a pure alkaloidal residue. Coloring agents were present together with some fatty materials which were difficultly soluble. The pure alkaloids from cinchona

TABLE II.—LIST OF SUBLIMATION TEMPERATURES OF THE VARIOUS FRACTIONS OBTAINED FROM CINCHONA AND NUX VOMICA AFTER TREATMENT WITH SODIUM CARBONATE

Plant	Number of Fractions	Sublimation Temperatures of Each Fraction at 5 mm., °	Sublimation Temperatures of Corresponding Alkaloids, °	
Cinchona	4	140–160	Quinine	140
		145–160	Quinidine	145
		145–160	Cinchonidine	145
		155–175	Cinchonine	155
Nux vomica[a] (defatted)	2	160	Brucine	160
		170–180	Strychnine	165–175

[a] Defatted with petroleum ether.

Quantitative Determination of Total Alkaloids in Cinchona by Sublimation and by the N. F. VIII Method.—A sample of red cinchona was reduced to a No. 60 powder in a Wiley Mill and dried in a desiccator over calcium chloride. A sample of known weight was taken and mixed with a known weight of anhydrous sodium carbonate in a ratio of three to one. Sufficient distilled water was added to wet the mixture which was then triturated in a mortar. The mixture was permitted to dry in a desiccator which was warmed to facilitate drying. The dried mixture was again reduced to a No. 60 powder and a sample of approximately 50 mg. was placed in the

in the quantities used, were found to be readily soluble in alcohol. A sample from these combined assay residues was weighed and a purification procedure attempted.

The residue was dissolved in a 2% solution of sulfuric acid and treated with activated charcoal. This mixture was filtered to remove some insoluble portions and contaminating coloring materials. The filter and charcoal were washed until no test was obtained on the washing with Mayer's reagent. The filtrate was made alkaline with sodium hydroxide T. S. The precipitated alkaloids were extracted with chloroform, and the combined chloroform solutions

TABLE III.—COMPARISON OF PERCENTAGES OF TOTAL ALKALOIDS IN CINCHONA DETERMINED BY THE SUBLIMATION METHOD AND THE N. F. VIII ASSAY

	Sublimation Method, %	N. F. VIII Assay, %
1	3.11	4.02
2	3.37	3.73
3	3.24	3.87
4	3.27	3.77
5	...	3.95
6	...	3.95
Av.	3.25	3.88

were evaporated to dryness in a tared vessel. The residue obtained was weighed.

In order to compensate for any losses due to this method, a blank sample was run with a 100-mg. sample of quinine alkaloid. The quinine was subjected to the same procedure; i.e., charcoal treatment followed by chloroform extraction. A loss of 5 mg. was obtained.

This correction was applied to a 233-mg. sample from the N. F. VIII cinchona assay residue. A loss in weight of 36 mg. was obtained after purification by the above procedure. The new residue was white and had a definite crystalline appearance, whereas the original residue from the N. F. VIII assay was colored, the crystals were indistinct, and mixed with a fatty material which could not be removed by the one purification procedure listed in the N. F.

The percentages of total alkaloids obtained by the N. F. VIII assay were corrected for the percentage of impurities present. An average of 3.28% total alkaloids was obtained. This compared very closely to the average obtained by direct sublimation.

Quantitative Determination of Strychnine in Nux Vomica by Microsublimation and by the N. F. VIII Method.—A sample of No. 60 nux vomica powder, previously defatted with petroleum ether, was mixed with anhydrous sodium carbonate in an exact ratio of four to one. The mixture was treated the same as cinchona mixture in the assay described above; that is, it was wetted and then dried.

A sample of approximately 50 mg., accurately weighed on a microbalance, was placed in a sublimation tube and heated in an aluminum drying block at a pressure of 5 mm. of mercury. The temperature was gradually raised until 160–165° was reached. A very small amount of sublimate was obtained at the sublimation temperature of brucine. This was discarded and the temperature was raised to 175–185°, at which range a crystalline sublimate of strychnine was obtained very rapidly. The sample was transferred quantitatively to a new sublimation tube and reheated under the same conditions. The process was continued until no further sublimate was obtained on examination of the tube microscopically.

The combined sublimates were washed out of the tubes with chloroform into a tared 5-cc. beaker and evaporated to dryness. The residue, previously dried to constant weight at 100°, was weighed on a microbalance.

The same sample of nux vomica was also assayed by the N. F. VIII method (8). Table IV shows the results of both assays.

TABLE IV.—COMPARISON OF PERCENTAGES OF STRYCHNINE IN NUX VOMICA DETERMINED BY THE SUBLIMATION METHOD AND THE N. F. VIII ASSAY

	Sublimation Method, %	N. F. VIII Assay, %
1	1.26	1.33
2	1.22	1.28
3		1.26

SUMMARY

1. It has been shown that a microsublimation technique can be used for the qualitative and quantitative separation of the alkaloids found in nux vomica and cinchona.

2. When the alkaloids are present as salts in the plant they must first be converted to the base before sublimation can be used to separate them. This is accomplished by treating the sample with sodium carbonate.

3. A microsublimation procedure has been described for the quantitative determination of the alkaloidal content of two plant drugs. Using nux vomica and cinchona, it was shown that the microsublimation assay procedure gave satisfactory results. When compared with the present official procedures, it was demonstrated that the results are more nearly a true determination of the alkaloids present.

REFERENCES

(1) Hubacher, M., *Ind. Eng. Chem., Anal. Ed.*, **15**, 448 (1943).
(2) Hoffman, H., and Johnson, W. C., *J. Assoc. Offic. Agr. Chemists*, **13**, 367(1920).
(3) Hubacher, M., *Ind. Eng. Chem., Anal. Ed.*, **15**, 448 (1943).
(4) Ray, P. C., and Datta, R. L., *J. Chem. Soc.*, **99**, 1475 (1911).
(5) Pregl, F., and Fyleman, E., "Quantitative Organic Microanalysis," ed. 2, The Blakiston Co., Philadelphia, 1930, p. 266.
(6) Benedetti-Pichler, A., *Ind. Eng. Chem., Anal. Ed.*, **2**, 309, 310(1930).
(7) "The National Formulary," ed. 8, Mack Printing Co., Easton, Pa., 1946, p. 147.
(8) *Ibid.*, p. 359.

The Precision of the U. S. P. Assay for Protamine Zinc Insulin*

By C. I. BLISS

The precision of the U. S. P. assay of protamine zinc insulin has been tested statistically with the data from duplicate assays in different laboratories of 24 preparations of protamine zinc insulin. Both the mean difference of six periodic bleedings and the trend of these differences with time were significantly greater than the residual or sampling error. The mean differences from two independent assays of the same preparation were not correlated. For these reasons, within the tolerance limits provided by the U. S. P., the mean difference could be attributed to minor variations in laboratory procedure rather than real differences in potency. Since the trend with time was correlated significantly in parallel assays from different laboratories, the trend must represent, at least partially, a real characteristic of a given preparation of protamine zinc insulin. It should appear that the assay for this insulin form is capable of distinguishing finer differences than it is called upon to make in actual practice.

PROTAMINE zinc insulin is characterized by its prolonged action and the U. S. Pharmacopœial assay is designed to test this effect as well as its average potency. In accord with the Food, Drug, and Cosmetic Act, each new batch is assayed against a specially prepared standard preparation in two or more rabbit cross-over tests in two different laboratories. It must pass both tests before it can be released. To determine the precision of the assay technique, paired assays of 24 preparations have been analyzed statistically. These data have been provided through the U. S. P. Insulin Advisory Committee and the present report has been prepared at their request.

The U. S. P. XIII assay may be outlined in brief. Each rabbit is injected twice, once with the standard preparation and once with the preparation under assay or the "unknown." An interval of about one week separates the two injections. In order to cancel out the effect of environmental factors, half of the rabbits are injected first with the standard and the other half first with the unknown. Immediately before each injection and periodically thereafter a sample of blood is taken from the ear vein of the rabbit and its glucose content analyzed. In the present experiments six bleedings were made at intervals of $1\frac{1}{2}$, 3, 5, 7, 9, and 11 hours after injection.

The difference in the blood-sugar level at each interval is determined by subtracting the average blood sugar observed in the tests with the unknown from that observed in the tests with the standard. Thus the responses are reduced to a series of seven differences, one representing the initial bleeding just before injection and six the bleedings which follow the injection of protamine zinc insulin. The Pharmacopœia requires that at each bleeding time after injection the difference must not exceed 5 mg. per 100 cc. except that at the last bleeding it may be as great as 8 mg. per 100 cc. There is the further requirement that the average of the six differences after injection, taking into account the sign of each difference, must not exceed limits of ± 3 for an acceptable preparation.

ANALYSIS OF ASSAYS

Individual Assays and Their Analysis.—The data obtained in paired assays of three preparations of protamine zinc insulin are summarized in Table I.

Each preparation was assayed in one laboratory (A_1, A_2, or A_3) and the assay repeated independently in testing Laboratory B. None of the individual differences (y) exceeded the above tolerances. Moreover, each total difference $S(y)$ was less than $\pm 3 \times 6 = \pm 18$. Hence these preparations conformed to U. S. P. standards. We may inquire whether the present biological assay is sufficiently precise that any characteristics of individual preparations which pass the U. S. P. requirements can be recognized from the agreement between independent paired assays. This can be tested statistically by an analysis of variance.

A simple analysis of variance may be computed from the six post-injection differences in blood-sugar level for each assay, such as those shown in Table I. It would test two null hypotheses. The first hypothesis is that the total (or mean) difference does not differ significantly from zero when compared with the residual variation. The second is that the differences do not become progressively larger or smaller in successive bleedings. Both hypotheses may be tested by fitting a straight line to the data of each assay, relating the difference in blood-sugar to the time of bleeding. The position of each line depends

* Received May 26, 1949, from the Connecticut Agricultural Experiment Station and Yale University, New Haven, Conn.

TABLE I.—DIFFERENCES IN MEAN BLOOD SUGAR (STANDARD - UNKNOWN) AT EACH BLEEDING FOR REPRESENTATIVE ASSAYS OF THREE PREPARATIONS OF PROTAMINE ZINC INSULIN

Prepn. No.	Testing Lab.	No. of Rabbits	Differences (y) in Blood Sugar at Hour 0	1½	3	5	7	9	11	Total Difference,[a] $S(y)$	Trend, $[xy]$[a]
3	A_1	41	1.0	−3.0	−2.0	0.0	2.0	1.0	−1.0	− 3.0	19.70
	B	32	0.0	0.1	−1.8	0.0	−3.6	−0.6	1.2	− 4.7	6.14
7	A_2	86	−0.6	−3.3	−0.5	−1.2	1.6	1.6	4.7	2.9	47.63
	B	31	−0.3	−3.8	−2.9	2.1	−1.1	1.0	7.9	3.2	65.57
22	A_3	38	−0.2	3.7	4.8	3.9	2.8	−2.2	1.9	14.9	−30.55
	B	27	1.3	0.6	−1.3	−1.9	1.3	−0.9	−4.9	− 7.1	−22.58
Deviation from mean hour after injection		x	...	−4.6	−3.1	−1.1	0.9	2.9	5.0	0	66.20 = $[x^2]$

[a] Excluding the initial reading (0 hr.).

upon the total difference $S(y)$ in the next to the last column of Table I. Its slope is computed by multiplying each difference (y) by the corresponding deviation (x) from the mean hour (last line of the Table). The sum of these products, $[xy]$, is shown for each assay in the last column. Each of these values is divided by $[x^2] = 66.20$ to obtain the slope of the line or trend.

From the values in Table I we obtain by standard methods the analyses of variance in the columns of Table II headed $[y_A^2]$ and $[y_B^2]$ for the two assays of each of the three preparations. The mean square for the "remainder" is an estimate with four degrees of freedom of the random or error variance in each assay. Each mean difference or trend may be tested for significance from the ratio (F) of its mean square or variance to that for the remainder. By this "F" test two trends and one mean difference were statistically significant, as indicated in Table II.

Composite Analysis of 24 Paired Assays.—The procedure illustrated in Tables I and II has been extended to all 24 preparations in the present series, representing a total of 48 assays. The corresponding sums of squares and products have been added for all preparations and the totals divided by the degrees of freedom to obtain the variances and covariances in Table III. The variances for the mean differences and for the trends with time of bleeding have been

TABLE II.—ANALYSIS OF COVARIANCE FOR THE PAIRED ASSAYS IN TABLE I IN UNITS OF THE DIFFERENCES IN TESTING LABORATORIES A (y_A) AND B (y_B), EXCLUDING INITIAL BLOOD SUGARS

Prepn. No.	Variation Due to	Degrees of Freedom	Variances and Covariances for $[y_A^2]$	$[y_A y_B]$	$[y_B^2]$
3	Mean difference	1	1.50	2.35	3.68
	Trend (slope)	1	5.86	1.83	0.57
	Remainder	4	2.91	− 2.47	3.44
7	Mean difference	1	1.40	1.55	1.71
	Trend (slope)	1	34.27[b]	47.18	64.95[a]
	Remainder	4	1.03	− 0.07	6.30
22	Mean difference	1	37.00[b]	−17.63	8.40
	Trend (slope)	1	14.10	10.42	7.70
	Remainder	4	4.28	− 1.98	4.02

[a] $P < 0.05$.
[b] $P < 0.005$ in test of significance.

Variability in the assay technique could be responsible for a significant mean difference or trend. Before attributing it to the characteristics of a given preparation of the drug rather than to the assay technique, we need to test the agreement between the two independent assays for each preparation. This requires the sums of products of the corresponding data from Laboratories A and B, paralleling exactly the sums of squares determined from the data of each laboratory. The means of these values or covariances are shown in the column of Table II under $[y_A y_B]$. When both the covariance and the adjacent variances are relatively large, the duplicate assays may well be correlated in the given characteristic. When small, any relationship is probably within the experimental error. Positive covariances indicate a positive relationship and negative covariances an inverse relationship between the paired assays. Of the three assays in Table II, the trend for preparation 7 was apparently similar in Laboratories A and B. The other values indicate random variation.

compared by the "F" test with the residual variance or error in the same column. Both factors varied significantly, in the three A laboratories (which have been considered together) and also in the control Laboratory B. The error variance for Laboratory B ($s^2 = 3.857$) was significantly larger (at $P < 0.05$) than that for the three laboratories included under the first column in Table III ($s^2 = 2.379$). Nearly all of this divergence could be explained by the fact that laboratory B used fewer rabbits than the other three laboratories (average of 31 against

TABLE III.—VARIANCES AND COVARIANCES FOR 24 PARALLEL ASSAYS OF PROTAMINE ZINC INSULIN IN LABORATORIES A_1–A_3 AND LABORATORY B. TRENDS WITH TIME WERE CORRECTED FOR THE EFFECT OF DIFFERENCE IN BLOOD SUGAR JUST PRIOR TO INJECTION BY A SEPARATE ANALYSIS OF COVARIANCE

Variation Due to	D. F.	Variances and Covariances for $[y_A^2]$	$[y_A y_B]$	$[y_B^2]$	Correlation, r, between A and B
Mean differences	24	7.243[b]	−1.110	8.184[a]	−0.144
Trends with time	23	8.554[b]	7.744	22.271[b]	0.561[a]
Remainder or error	95[c]	2.379	0.451	3.857	0.149

[a] $P < 0.01$.
[b] $P < 0.001$ in test of significance.
[c] One aberrant 7-hr. mean difference was replaced by interpolation from the curve fitted to the remaining values, reducing the degrees of freedom from 96 to 95.

50). Although the number per assay also varied considerably, especially among the three A laboratories, each assay was given unit weight without attempting to adjust for inequalities in the number of rabbits.

Although the mean difference and the trend with time varied more from zero than would be expected by chance, these might still represent assay errors rather than real differences between preparations. In the former case the results from paired assays in different laboratories should be uncorrelated but in the latter case a correlation would be expected. The ratio of the covariance term in each row of Table III to the square root of the product of the adjoining variances gave the correlation coefficients, r, in the last column.

The correlation of the mean differences was small and negative and far below the level required for significance. It seems evident therefore, that where the mean differences exceeded the random error, they represented the small deviations in average potency which are unavoidable in preparing a fresh standard preparation for each assay. The deviations around the trend lines fitted to each assay also were uncorrelated, as would be expected for the random errors in independent tests. The correlation of the trend with time, on the other hand, was clearly significant ($r = 0.561$). It is evident that the assay procedure was capable of detecting small trends in the blood-sugar response to commercial preparations. This occurred even though the test preparations were compared with a standard which was prepared independently in each laboratory and the trend was limited by the restrictions on the differences allowed in the periodic blood sugars. Thus the mean difference from the 6 bleedings was apparently associated with the assay technique but part of the trend on time was a characteristic of the preparation under assay.

In the assay of Insulin Injection it has been shown repeatedly that the percentage fall in blood sugar is related to the blood-sugar level immediately before injection. In the present experiments the difference in the initial blood-sugar level may have affected either the mean difference or the trend of the difference with time. The first possibility was tested by computing the regression of the total difference upon the initial level. No relation was observed in either the A or the B Laboratories. A similar regression of the trend upon the initial difference, however, showed that the trend depended to a very significant degree upon the initial level in Laboratories A_1 to A_3 and to a lesser extent in Laboratory B. In both cases the larger the initial difference the smaller was the slope or trend with time following injection. In consequence the variances and covariances in Table III for the effect of trend have been corrected by a separate covariance for difference in the initial blood-sugar level. This reduced the degrees of freedom in the second row of Table III from 24 to 23. However, the correlation in the trend between paired assays was practically unchanged by the adjustment.

SUMMARY

A statistical analysis of parallel assays in different laboratories of 24 preparations of protamine zinc insulin has tested the precision of the U. S. P. assay procedure. Both the mean difference over 6 periodic bleedings and the trend of these differences with time proved significantly greater than the residual or random sampling error in the two series of tests. The mean differences from two independent assays of the same preparation were not correlated. Hence within the tolerance limits provided by the Pharmacopœia the mean difference could be attributed to minor variations in laboratory procedure and not to real differences in potency. The trend with time, however, was correlated significantly in parallel assays from different laboratories. The trend, therefore, represents at least in part a real characteristic of a given preparation of protamine zinc insulin. Evidently, the U. S. P. method of assay for protamine zinc insulin is capable of distinguishing finer differences than it is called upon to make in practice.

The Sheep Plasma Method for the Bioassay of Heparin Preparations*

By ORLO F. SWOAP and MARVIN H. KUIZENGA

A detailed description of the sheep plasma method for the bioassay of heparin preparations is presented. The potency of unknown heparin is determined by a simultaneous comparison of the minimum amounts of standard and unknown heparin which, when contained in a volume of 0.3 cc., will keep 1 cc. of recalcified sheep plasma more than 50 per cent fluid for one hour at room temperature. The method is short, accurate, easy to use, and adaptable to any heparin preparation. Specific advantages over other methods may be listed as follows: the amount of calcium chloride necessary for recalcification of the plasma need be determined only once for each lot of frozen plasma. Tubes stand one hour and for approximate or spotting tests can be examined in fifteen to thirty minutes. No water bath is required. Twenty to twenty-five samples may be assayed in one working day by an experienced operator. Calculation of results is rapid and simple. The dosage response curve is steeper than those found with other methods or with other plasmas. The end point is easy to determine. Results can be determined with sufficient accuracy without plotting data obtained.

A NUMBER of bioassay methods have been proposed for testing heparin preparations. The most common methods can be divided into two classes and can be designated as (A), those using whole blood, and (B), those using citrated or oxalated plasmas. In class A, we find methods using cat blood as described by Howell (1), and modified by Charles and Scott (2), beef blood as described by Jorpes (3, 4), chicken blood as described by Fischer and Schmitz (5), and rabbit blood as described by Schutz (6). In class B, we find methods using beef plasma as described by Reinert and Winterstein (7), and Foster (8), horse plasma as described by MacIntosh (9), and sheep plasma as described by Kuizenga, *et al.* (10).

The disadvantages in the use of cat blood have already been reported from this laboratory (10). In general, the disadvantage of the routine use of any whole blood in an assay method is that it is practically impossible to collect the blood and complete the assay of a large number of samples in any one day. The use of citrated plasmas has the definite advantage that they can be collected in quantity as pooled samples from a number of animals, bottled so that the contents of one bottle will be sufficient for a day's test, frozen, and stored in the frozen state until used.

Since the original publication from this laboratory (10), we have repeatedly confirmed the previous conclusions reached as to the superior properties of sheep plasma over beef plasma. Several modifications have also been added to the method to increase its usefulness as a routine assay method. It is the purpose of this paper to present in detail the sheep plasma assay method for heparin which is in current use in our laboratories. We feel that the principal advantage of this method lies in its simplicity, adaptability, and ease of operation. The method is rapid, accurate, and easy to use, even when the operator has had no previous experience with the method. When one becomes familiar with the method, upward to 20–25 unknown samples can be tested in an eight-hour working day.

METHOD AND RESULTS

Details of the Sheep Plasma Method.—In this method the potency of unknown heparin is determined by a simultaneous comparison of the minimum amounts of standard and unknown heparin which, when contained in a volume of 0.3 cc., will keep 1 cc. of recalcified sheep plasma more than 50% fluid for one hour at room temperature.

Plasma.—Draw sheep blood directly from the animal into a sterile vessel containing 50 cc. of a 4% sodium citrate solution for each 500 cc. of blood. Mix the blood and sodium citrate solution by gentle agitation and separate the plasma by centrifugation. The clear, fresh plasma may be used at once for the assay described below, or it may be put in suitably sized bottles, so that the contents of one bottle will be sufficient for a day's test, frozen, and stored in the frozen state at −20° for future use.

The above frozen sheep plasma may be obtained from a packing house and stored in the frozen state at −20° for future use. Such plasmas have been stored for as long as a year without showing signs of deterioration.

Frozen plasma may be thawed for use by placing in a water bath at 37° until fluid. When fluid, strain through about two layers of 60-mesh cheese cloth, or through glass wool.

* Received May 27, 1949, from the Research Laboratories, The Upjohn Co., Kalamazoo, Mich.

Determination of $CaCl_2$ for Recalcification.—The optimum amount of calcium chloride necessary to bring about complete coagulation of the plasma in as short a time as possible is determined by placing 1-cc. samples of the strained sheep plasma in each of four test tubes and adding 0.05, 0.10, 0.15, and 0.20-cc. quantities of a 2% calcium chloride solution. (Three-tenths of a cubic centimeter of 0.9% NaCl solution may be added to each tube before adding the calcium chloride to be comparable to the assay procedure.) The optimum amount of calcium chloride solution is that which, in the shortest time, produces a firm clot that cannot be removed from the tube by inverting it. This optimum amount of calcium chloride is then used per cc. of that particular lot of plasma.

Preparation of Dilutions of Standard or Unknown Heparin.—All solutions or dilutions of heparin are to be made with isotonic (0.9%) sodium chloride.

A stock solution of the Provisional International Heparin Reference Standard may be prepared in 0.9% sodium chloride (a 1-mg./cc. solution is satisfactory). This solution must be kept in glass-stoppered containers or sealed ampuls. When tightly stoppered and refrigerated, such a solution will be stable for at least three months. Further dilutions are made with 0.9% sodium chloride immediately before use, so that the estimated minimum amount of standard heparin necessary to maintain more than 50% fluidity of the plasma will be contained in 0.3 cc. or less. For those who have had no previous experience with the method, it may be stated that this amount will probably fall within the range of about 1.0–1.5 "Toronto Units." A 1–20 dilution of the above stock solution will be satisfactory. This dilution would contain 0.05 mg. of the Provisional International Heparin Reference Standard per cc.

Preparation of Coagulation Tubes.—A series of test tubes (size 12 x 75 mm.) (previously cleaned in acid-dichromate solution, thoroughly rinsed, and dried) is set up for the standard, and for each unknown to be tested, and varying amounts of the heparin dilution are accurately measured into the tubes (for instance, 0.16, 0.18, 0.20, 0.22, 0.24, 0.26, 0.28, and 0.30 cc.). The amounts used are selected so that the minimum amount necessary to maintain fluidity of the plasma, as determined by preliminary trial, is approximately at the center of the series of tubes. The volume of the heparin solution in each tube is then made up to 0.3 cc. by adding 0.9% sodium chloride. (For the above-mentioned series, volumes of isotonic sodium chloride added to each tube would be 0.14, 0.12, 0.10, 0.08, 0.06, 0.04, 0.02, and 0.00 cc.)

The Anti-Coagulation Test.—To each of the above tubes, add 1.0 cc. of the strained sheep plasma and then, noting the time, add the optimum amount of calcium chloride solution as determined above. Immediately stopper each tube with a paraffined cork and mix the contents by inverting the tube three times. Keep the tubes thus prepared at room temperature, and exactly one hour after recalcification examine each tube for the extent of coagulation. As in all biological work the dosage response curve is S-shaped, with the steepest part of the curve giving the most accurate end point for comparison of standard and unknown. The previous report from this laboratory (10) has shown that sheep plasma gives a much steeper curve than do beef or horse plasmas and thus the end point is sharper. Those tubes showing complete clotting may easily be determined by inverting the tube. Completely fluid tubes can also be easily determined. Those tubes falling on the steep part of the curve are evaluated by estimating visually the amount of clot and fluid present (see Discussion). Because of the steepness of the dosage response curve, very few questionable tubes are encountered. Any tube showing partial clotting is shaken sufficiently to estimate whether the clot is more or less than 50% of the total contents of the tube. If the clot is greater than 50% of the total volume, the tube is called clotted. If the clot is less than 50% of the total volume, the tube is called fluid.

Evaluation of Results.—The end point is taken as the minimum amount of heparin necessary to maintain fluidity of the plasma (i.e., less than 50% clot) and this value is recorded for both standard and unknown. From these values the potency of unknown in terms of the standard is determined. For example, if tube No. 4 of the standard series, containing 0.22 cc. of a 0.05 mg./cc. solution of this standard (= 0.011 mg. of standard), contains the minimum amount of this standard necessary to maintain >50% fluidity (or <50% clot), this value is recorded as the end point for the standard.

Powdered Samples

If tube No. 5 of the unknown series, containing 0.24 cc. of a 0.05 mg./cc. solution, matches the above tube No. 4 of the standard series, then

$$\frac{0.011}{0.24 \times 0.05} \times \text{Potency of the Standard}^1 = \text{Potency of the Unknown}^2\text{/mg.}$$

Solutions

If tube No. 5 of the unknown series, containing 0.24 cc. of a 1–200 dilution of the unknown, matches the above tube No. 4 of the standard series, then

$$0.011 \times \frac{200}{0.24} \times \text{Potency of the Standard}^1 = \text{Potency of the Unknown}^3\text{/cc.}$$

DISCUSSION

The above-suggested method uses increments of 1 γ of standard heparin, the total amounts in each tube differing by approximately 10%. If desired, smaller increments of dosage may be used in a series of tubes, but accuracy of the test will depend on the care and accuracy of measuring these volumes. Accuracy of a determination may be increased by running two or more series of tubes for each sample, and averaging the results obtained.

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[1] 130 units/mg. for the Provisional International Heparin Reference Standard.
[2] 119.2 units using the Provisional International Heparin Reference Standard.
[3] 1192 units using the Provisional International Heparin Reference Standard.

In measuring such small volumes as are used in this assay, one may consider that accuracy can be obtained only by using a microburette for the measurements of volume. However, if as many as 20–25 unknown samples are being tested in one day, the calibration of each burette used and the cleaning and preparation of these burettes becomes a major factor and sacrifices speed or limits the number of samples that can be tested at once to a smaller number. To attain speed and ease of operation, we wish to point out that in this assay we are comparing standard and unknowns diluted to approximately equal potencies. A good technician can easily measure the volumes of dilutions and of saline from a calibrated pipette with a high degree of accuracy. The same pipette may be used for measuring the next dilution after first rinsing with that dilution. This is a major factor in the ease of operation of the test and experience has proved that accuracy is not sacrificed to any significant extent. Automatic measuring pipettes or microburettes may be used with advantage in measuring the plasma and calcium chloride solutions.

We are in agreement with Foster (11) that the end point is sharper when the calcium chloride, used for recalcification of the plasma, approximates the stoichiometrical equivalent of the sodium citrate used in preparation of the plasma.

Various reports have appeared in the literature on the determination of the percentage of clot produced in tubes used in the plasma assays of heparin. In our opinion such methods, though adding to the accuracy of many methods, are not necessary for use with a dosage response curve as steep as found with sheep plasma in the above-described assay procedure. The method as originally presented (10) pointed out the steepness of this curve. Because of the steepness of this curve and the recording of results as clotted or fluid only, the method has been referred to as an all-or none-response method (11). We have received comments from other laboratories that the method could be improved by setting the end point at 50% clot. As pointed out above in the details of the method, our end point is at the 50% clot level or the first tube below it. Accuracy is increased by reducing the increments between tubes to approach this end point. The steepness of the dosage response curve gives accuracy by this method without drawing the actual curve for each sample and determining the dose which would produce the 50% clot as suggested by Foster (8). Foster's method of calculation of results, or other suggested methods of estimating clot formation, are more time-consuming and it is questionable whether the data obtained in this type of assay warrants such elaborate treatment, where an assay for routine testing of a large number of samples is desired.

The sheep plasma method as previously reported (10) and here modified has been used by several other laboratories and cooperative work between these laboratories and our own has proved workability and accuracy of the method. Comparison with other methods has shown our procedure to give results as accurate as those obtained with the other methods. In addition, it is more suitable for routine use because of the speed, adaptability, and ease of operation.

SUMMARY

We have presented in detail the sheep plasma method for the assay of heparin preparations which has been briefly described in an earlier publication from this laboratory (10).

The method is short, accurate, easy to use, and adaptable to any heparin preparation. Specific advantages over other methods may be listed as follows: The amount of calcium chloride necessary for recalcification of the plasma need be determined only once for each lot of frozen plasma. Tubes stand one hour and for approximate or spotting tests can be examined in fifteen to thirty minutes. No water bath is required. Twenty to twenty-five samples may be assayed in one working day by an experienced operator. Calculation of results is rapid and simple. The dosage response curve is steeper than those found with other methods or with other plasmas. The end point is easy to determine. Results can be determined with sufficient accuracy without plotting data obtained.

REFERENCES

(1) Howell, W. H., *Am. J. Physiol.*, **71**, 553(1925).
(2) Charles, A. F., and Scott, D. A., *Biochem. J.*, **30**, 1927(1936).
(3) Jorpes, J. E., *ibid.*, **29**, 1817(1935).
(4) Jalling, O., Jorpes, J. E., and Linden, G., *Quart. J. Pharm. Pharmacol.*, **19**, 96(1946).
(5) Fischer, A., and Schmitz, A., *Z. physiol. Chem.*, **210**, 129(1932).
(6) Schutz, F., *Quart. J. Pharm. Pharmacol.*, **14**, 45(1941).
(7) Reinert, M., and Winterstein, A., *Arch. Intern. pharmacodynamie*, **62**, 47(1939).
(8) Foster, R. H. K., *J. Lab. Clin. Med.*, **27**, 820(1942).
(9) MacIntosh, F. C., *Biochem. J.*, **35**, 770(1941).
(10) Kuizenga, M. H., Nelson, J. W., and Cartland, G. F., *Am. J. Physiol.*, **139**, 612(1943).
(11) Foster, R. H. K., THIS JOURNAL, **36**, 243(1947).

A Method for the Evaluation of Radiographic Opaques*

By CHARLES H. SPRAGUE†, H. GEORGE DEKAY, GLENN L. JENKINS, and JOHN E. CHRISTIAN

A method for the preliminary evaluation of radiographic opaques *in vitro* has been developed. The method employs a device holding X-ray film, photometric absorption cells containing opaque media, and aluminum strips of varying density as reference standards. The method eliminates sources of error such as thickness of solution layer and exposure and developing times. No relationship between chemical structure and opacity was found in a series of compounds. A relationship between chemical composition and opacity has been found.

THE OBJECTIVE of this study was to devise a method of accurately measuring the X-ray densities of opaque media *in vitro* for the analytical evaluation of their comparative opacities.

A survey of the literature for methods of evaluation of radiologic opaques *in vitro* revealed that only two techniques have been reported, and neither seems sufficiently accurate or simple to serve our purpose. The method employed by Binz, *et al.*, involved the use of a metal vessel which contained the opaque in solution. Two glass-covered openings in opposite sides of the container allowed X-rays to pass through the opaque solution and fall upon an X-ray film. The film was developed and the density of a darkened spot thus produced on it was measured by a photometric device and taken as an index of efficiency of the opaque (7). Joffe and Wachowski used rubber bags 3.7 cm. in diameter containing 30 cc. of bile to which the opaque compound was added. These bags were then placed in a water phantom and X-rayed (8).

Examination of the first method revealed several places where errors might be introduced. The thickness of the opaque solution, the exposure time, and the time of development of the film all enter as factors that might vary the results. Likewise in the second method, the thickness of the opaque solution and the exposure time as well as the varying thickness of the rubber of the bags might cause variation in density of the exposure on the X-ray film.

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APPARATUS

A new device and technique have been developed for testing and comparing opaques. The parts and materials used included the following:

Film and Solution Holder.—The apparatus constructed to hold the X-ray film and opaque solution containers was made entirely of wood. All joints were glued, thus eliminating any metallic objects other than the metallic strips used as density reference standards.

Absorption Cells.—Central Scientific Company catalogue No. 12336-A absorption cell. These containers were used to hold the opaque media. By the use of these cells, which have optically flat sides, a constant thickness of solution of 1 ± 0.005 cm. was obtained. The apparatus was designed to hold 12 of these cells. Since the pictures of several opaque solutions are obtained on one film, the solution thickness, exposure and developing times remain constant. Thus the sources of error that might have been encountered in the methods cited previously are eliminated.

Density Reference Standards.—Aluminum strips 7 inches long, 1/8 inch wide and 0.008, 0.022, and 0.062 inch thick. The aluminum strips were inlaid vertically in the plywood backing between the solution cells and the film holder. Four sets of a series of three thicknesses of aluminum strips were placed behind each of the four vertical rows of cells. The final setup is illustrated in Fig. 1.

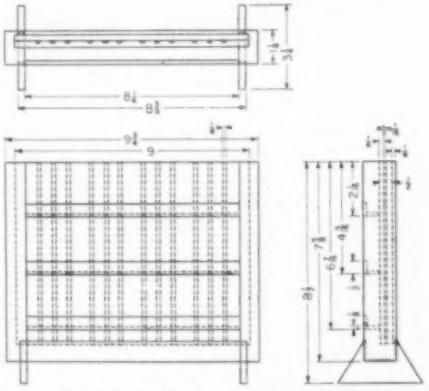

Fig. 1.—The broken vertical lines in the front view of the drawing indicate the position of the metallic strips used as density reference standards. The side view shows the shelves on which the absorption cells containing opaque solutions are placed. The top view shows the position of the film holder behind the slots for the metallic strips. All dimensions are in inches.

X-ray film. Eastman Kodak Co. Blue-Brand duplitized X-ray film.
Developer. Kodak liquid X-ray developer and replenisher.
Fixer. Hypo with chrome alum sulfite hardener.
Opaques. A variety of opaques were used to test the method.
Iodine solution.
Phentetiothalein sodium N. N. R. Mallinckrodt Chemical Company.
Hippuran N. N. R. Mallinckrodt Chemical Company.
Skiodan. Winthrop Chemical Company.
Diodrast. Winthrop Chemical Company.
Lipiodol Lafay. E. Fougera and Company.
Lipoiodine. Ciba Pharmaceutical Products Company.
Neo-iopax. Schering Company.
Priodax. Schering Company.

PROCEDURE

X-ray photographs of aqueous iodine solutions were first made. In order to determine the concentration of iodine per cc. necessary to blank out the various thicknesses of aluminum density reference standards, the following general procedure was carried out.

A solution containing 300 mg. iodine per cc. was made by dissolving 50 Gm. KI and 37.5 Gm. iodine in 250 cc. of solution. By dilution of this solution the following concentrations of iodine were obtained: 280, 260, 240, 220, 200, 180, 160, 140, 120, 100, 80, and 60 mg. iodine per cc.

Aluminum metal strips used: 0.008, 0.022, and 0.040 inch.

The solutions were placed in the holder in descending order of concentration from left to right with the most concentrated in the upper left and the least concentrated in the lower right.

The film was exposed for one-tenth of a second at a 30-inch focal film distance and 46 kv. p. using a 100-milliamp. tube. The results are shown in Fig. 2.

X-ray photographs of the various opaque solutions were made according to the general procedure as follows:

Solutions of various opaques were made to contain 180, 140, and 100 mg. iodine per cc. The concentrations of opaque necessary to obtain these values are shown in Table I. The solutions were aqueous with the exception of Lipoiodine and Lipiodol which were diluted with peanut oil. The concentrations of thorium nitrate, bismuth nitrate, cadmium nitrate, and mercuric chloride solutions used were calculated to Hull's law, cited by Greenbaum and Peters, which states that the opacity of an element is proportional to the third power of its atomic weight (4). Each of the opaque solutions was then compared with iodine solutions of the same concentration of iodine, 12 samples being exposed to X-rays in the holder at the same time. The left vertical row of three solutions consisted of the iodine solutions, the 180 mg./cc. solution being in the top holder, the 140 mg./cc. solution in the middle holder, and the 100 mg./cc. being in the bottom holder. Three other opaques were compared at the same time, and arranged in the same order of concentration vertically.

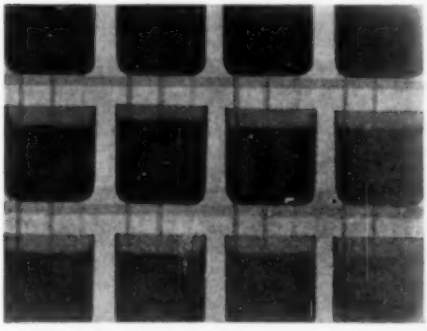

Figure 2

Aluminum metal strips used: 0.008, 0.022, and 0.062 inch.

The film was exposed for one-tenth of a second at a 30-inch focal film distance and 46 kv. p. using a 100-milliamp. tube.

The photographs were all made in the same period and developed simultaneously. The development data for all films were as follows: time in developer, three and one-half minutes at 68° F.; time in fixer, seven minutes at 68° F.; washed twenty minutes at 68° F.

DISCUSSION

The search for a proper density reference standard involved many trials of various materials. Various thicknesses of aluminum were tried and those 0.008, 0.022, and 0.062 inch thick were selected as the ones that gave the desired degree of contrast.

From the results it was shown that 160 mg. I_2 per cc. blanked out the 0.008- and 0.022-inch aluminum strips, while with 140 mg. I_2 per cc., the 0.022-inch aluminum strip was discernible. Because the 140 mg. I_2 per cc. was on the border of blanking out the 0.022-inch aluminum strip it was chosen as one of the reference control solutions and the other two arbitrarily selected were 180 and 100 mg. I_2 per cc.

The results of the experiments showed that all of the compounds containing iodine in concentrations giving the same concentration of iodine as the control gave the same degree of opacity as did the iodine control. However, in the metallic solutions the results obtained were not those expected since thorium, bismuth, and mercury did not blank out the 0.022- and 0.062-inch aluminum strips even in the most concentrated solutions while the cadmium blanked them out in the least concentrated solution. This gives doubt to the validity of Hull's law as stated by Greenbaum and Peters (4).

This method is being used to study the relative opaque efficiency of various materials in an effort to relate chemical constitution and structure with opacity.

CONCLUSION

A new method for evaluating chemical compounds for their radiographic opaque properties has been devised. Sources of error such as thickness of solution, exposure and developing times have been eliminated. The relationship between chemical structure and opacity and between chemical composition and opacity may be conveniently studied using this method.

TABLE I.—CONCENTRATIONS OF OPAQUES NECESSARY TO OBTAIN SOLUTIONS OF 180, 140, AND 100 MG IODINE PER CC.

Opaque	Iodine Content	Gm. or Cc. per 15 Cc. Solvent Equivalent to—		
		180 Mg. I_2/Cc.	140 Mg. I_2/Cc.	100 Mg. I_2/Cc.
Lipiodol	0.54 Gm. per cc.	5 cc.	3.89 cc.	2.77 cc.
Lipoiodine	41.0%	6.58 Gm.	5.12 Gm.	3.65 Gm.
Hippuran	34.9%	7.72 Gm.	6.00 Gm.	4.30 Gm.
Neo-iopax	51.5%	5.24 Gm.	4.07 Gm.	2.91 Gm.
Diodrast	50.0%	5.40 Gm.	4.20 Gm.	3.00 Gm.
Skiodan	52.0%	5.19 Gm.	4.04 Gm.	2.90 Gm.
Phentetiothalein	58.6%	4.90 Gm.	3.80 Gm.	2.71 Gm.
Thorium nitrate	...	1.07 Gm.	0.83 Gm.	0.59 Gm.
Mercuric chloride	...	0.93 Gm.	0.72 Gm.	0.52 Gm.
Bismuth nitrate	...	1.40 Gm.	1.09 Gm.	0.78 Gm.
Cadmium nitrate	...	10.65 Gm.	8.28 Gm.	5.92 Gm.

REFERENCES

(1) Green, A. B., *X-ray Technician*, **9**, 231(1938).
(2) Sicard, J. A., and Forestier, J. E., *Radiology*, **7**, 385 (1926).
(3) Mettier, S. R., and Leake, C. D., *J. Am. Med. Assoc.*, **111**, 986(1938).
(4) Greenbaum, F. R., and Peters, A. F., *Radiology*, **35**, 45(1940).
(5) Baker, W., Cook, E. E., and Leeds, W. G., *J. Soc. Chem. Ind.*, **63**, 223(1944).
(6) Tarlov, I. M., *J. Am. Med. Assoc.*, **129**, 1014(1945).
(7) Binz, A., Rath, C., Maier-Bode, H., and Hermann, K., *Angew. Chem.*, **45**, 713(1932).
(8) Joffe, H., and Wachowski, T. J., *Radiology*, **38**, 43 (1942).

Tryptophan as Solubilizing Agent for Riboflavin*,†

By ROBERT A. HARTE‡ and JAMES L. CHEN§

Stable solutions of riboflavin, suitable for injection, may be prepared by taking advantage of the effect of *l*- or *dl*-tryptophan on increasing the solubility of the vitamin. Solutions combining the effects of niacinamide and tryptophan on riboflavin solubility contain up to 7 mg. per cc. of riboflavin and 100 mg. per cc. of niacinamide. They are physically stable and retain biological potency for periods up to at least seven months as determined by photofluorometric and animal tests.

THE IMPORTANCE of finding physiologically innocuous agents to enhance the solubility of riboflavin in aqueous media is amply attested by the numerous publications on this subject and by the large number of patents which have been issued (1). None of these processes seem to have proved entirely satisfactory and work in this field continues. The evidence of Strong, *et al.* (2), suggests that the attainment of concentrations of about 5 mg. of riboflavin per milliliter may be a satisfactory goal.

This report deals with the preparation of solutions of approximately such concentrations of riboflavin by using tryptophan as solubilizing agent. Solubility determinations have been made in systems whose *p*H was controlled by appropriate buffers.

EXPERIMENTAL

Preliminary tests.—Concentrated aqueous solutions of the naturally occurring enantiomorphs of the following amino acids failed to dissolve appreciably more riboflavin than water alone: alanine, arginine, aspartic acid, cysteine, cystine, glutamic acid, histidine, hydroxyproline, leucine, lysine hydrochloride, proline, serine, threonine, tryosine, and valine. In addition, glycine, *dl*-methionine, and monosodium glutamate were tried and not found useful for this purpose. On the other hand, both *l*- and *dl*-tryptophan were found to possess definite solubilizing effects on riboflavin.

Preparation of Solutions.—Solutions were prepared by shaking an excess of riboflavin with the desired solvent for ten minutes at 70° to 75° in a water bath and filtering after cooling to room temperature. Alternatively, solutions were prepared by heating mixtures of the substances with water or buffer solutions in sealed tubes in a boiling water bath for several hours or filtration completed after longer standing at refrigerator temperatures, or both.

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† The authors wish to thank J. J. Travers and P. Sarich for their cooperation in carrying out the biological tests.
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§ Present address: E. R. Squibb and Sons, Brooklyn, N. Y.

Since *l*-tryptophan is about four times as soluble in water as the *dl*-isomer (3) (1.14% at 25° as compared to 0.25% at 30°, respectively), it would be expected that saturated solutions of the former would dissolve more riboflavin than similar solutions of the latter, unless the steric configurations exert an influence. Actually, at *p*H 6.8 (MacIlvaine's buffer) the solubility of riboflavin in saturated *l*-tryptophan solution was about 4 mg. per ml.; with *dl*-tryptophan, about 2.2 mg. per ml.

When the solutions were prepared by heating excesses of the amino acid and riboflavin in buffers of various *p*H's (MacIlvaine's citric acid–sodium phosphate system), cooling in the refrigerator (5–8°), and filtering, the solubility of riboflavin seemed related to the *p*H and the varying salt contents of the mixtures; possibly a maximum concentration of riboflavin is obtained at about *p*H 3.2 (4.4 mg. per ml.). See Table I.

TABLE I.—SOLUBILITY OF RIBOFLAVIN IN SATURATED *l*-TRYPTOPHAN SOLUTIONS IN CITRIC ACID–SODIUM PHOSPHATE BUFFERS

*p*H	Riboflavin Mg. per Cc.
2.8	3.3
3.2	4.4
4.0	2.7
6.8	4.0

Solvents prepared by dissolution of tryptophan in 10% niacinamide solutions were capable of dissolving up to 7 mg. of riboflavin per ml. Such a solution had a *p*H of 6.3. This concentration may be greater than what would be expected if the effects of niacinamide and tryptophan on the solubility of riboflavin were merely additive. However, the presence of niacinamide substantially increases the solubility of tryptophan and this observation may explain the apparent synergy observed in these experiments.

Chemical Stability.—A variety of solutions containing combinations of tryptophan and niacinamide saturated with riboflavin, at *p*H's up to 6.3, were examined after seven months storage at ambient temperatures in dark bottles. All of these solutions remained clear. Photofluorometric assays for riboflavin content checked well with the initial assay values.

Biological Adequacy.—Animals were maintained on synthetic rations similar to those previously reported from these laboratories (4), except that, where appropriate, riboflavin was omitted from the vitamin supplements. The test rations contained 17% vitamin-free casein and 0.18% choline chloride. Four groups of eight weanling males were used Two groups (A and B) received the ration containing 8 mg.–Kg. of riboflavin and two groups (C and D) received riboflavin-free rations.

All animals received daily injections of 0.1 ml.: the injected solutions being, for Groups A and C, physiological saline; for Group D, an appropriate dilution of a 10% niacinamide solution saturated, with respect to *l*-tryptophan and riboflavin (7 mg. per ml.); and Group B, a corresponding dilution of a 10% niacinamide solution saturated, with respect to *l*-tryptophan.

After four weeks, the animals in Groups A, B, and D showed comparable weight gains of about 100 Gm; Group C animals (negative controls) gained only about 20 Gm. At this time, half of the negative control animals were given daily injections of the niacinamide-tryptophan-riboflavin solution. After seven weeks, studies on the remaining negative control animals which had lost weight were discontinued. Since the animals in Groups A and B performed virtually identically, surveillance of Group A was discontinued after seven weeks. Animals in Groups B and D continued to show comparable weight gains while the negative control animals (Group C) which had begun to receive riboflavin injections on the twenty-eighth day slowly caught up with those receiving riboflavin regularly from the beginning of the experiment. The amount of riboflavin injected was increased to keep pace with the greater intake in Group B as these animals grew larger. After twenty weeks the experiment was terminated. The animals in Group B had a total riboflavin intake of 14.6 mg. during the test; Group B had a total riboflavin intake of 14.6 mg. during the test; Group D, 14.2, and Group C, 11.9 mg. Analysis of variance showed no significance difference between the weight gains of any of the groups. Postmortem examination of the sacrificed animals revealed no gross evidence of liver or kidney damage, which might have been expected had any methyl deficiency been induced by the stress of excretion of the relatively massive doses of niacinamide as its methylated metabolic end product.

DISCUSSION

The previous literature on solubilizing agents for riboflavin has been summarized by Frost (1). To it may be added Bird and Kuna's patent (5) dealing with *l*-tyrosinamide and its salts and the report of Schoen and Gordon (6) on methylol derivatives of riboflavin. The unphysiological nature of some of the solubilizing agents previously advocated is only too evident and does not require emphasis here. Although niacinamide, which has been used extensively by the pharmaceutical industry, is an entirely physiological substance, nevertheless the riboflavin-niacinamide ratio necessary to obtain the desired concentration of riboflavin would not appear desirable. The entirely physiological nature of tryptophan, together with its well-established metabolic relationship to niacin or niacinamide (7, 8), makes it a potentially useful addend as a solubilizing agent for riboflavin. Apparently, too, *dl*-tryptophan is not toxic when administered orally to humans in quantities of 5 Gm. daily (9).

SUMMARY

1. Concentrated aqueous solutions of *l*- or *dl*-tryptophan dissolve more riboflavin than does water.

2. The effect of such solutions on the solubility of riboflavin seems to be related to the concention of the amino acids.

3. Riboflavin in such tryptophan solutions dissolves to the extent of 4 mg. per ml. at *p*H 6.8.

Slightly higher concentrations were observed at *p*H 3.2, perhaps due to different salt concentration.

4. Addition of niacinamide markedly increases the solubility of riboflavin as a reflection, in part, of its solubilizing effect on tryptophan.

5. Such solutions of riboflavin have been shown to remain clear and stable over a seven-month period of storage at room temperature.

6. The biological efficacy of such solutions has been demonstrated by a twenty-week growth study with weanling rats.

REFERENCES

(1) Frost, D. V., *J. Am. Chem. Soc.*, **69**, 1064(1947).
(2) Strong, F. M., Feeney, R. E., Moore, B., and Parsons, H. T., *J. Biol. Chem.*, **137**, 363(1941).
(3) Howe, E. E., personal communication.
(4) Harte, R. A., Travers, J. J., and Sarich, P., *J. Nutrition*, **36**, 667(1948).
(5) Bird, J. C., and Kuna, A., U. S. Patent 2,407,624 Sept. 17, 1946.
(6) Schoen, K., and Gordon, S. M., "Abstracts of Papers, 114th Meeting, American Chemical Society," p. 16K, 1948.
(7) Heidelberger, C., Gullberg, M. E., Morgan, A. F., and Lepkovsky, S., *J. Biol. Chem.*, **175**, 471(1948).
(8) Heidelberger, C., Abraham, E. P., and Lepkovsky, S., *ibid.*, **176**, 1461(1948).
(9) Sarett, H. P., and Goldsmith, G. A., *ibid.*, **177**, 461 (1949).

Screening Tests of Certain New Organic Fluorine Compounds for Ascaricidal and Ascaristatic Activities*

By R. H. FODEN,† P. H. HONDA,† and L. D. EDWARDS‡

Results of screening tests of 24 organic fluorine compounds by the method of Lamson and Brown and by a modified form of Baldwin's method for ascaricidal and ascaristatic activities are given.

SODIUM FLUORIDE, a general protoplasmic poison, has been advocated as an ascarcide in swine (1). It was suggested that organic fluorine compounds might prove to be effective as ascaricides, and would be, since fluorine is rather firmly held by carbon, fluorine compounds of reduced general toxicity. The organic fluorine compounds studied in this report were prepared by Dr. E. T. McBee and his staff of the chemistry department of Purdue University. The method of Lamson and Brown (2) was used to screen these compounds for ascaricidal activity; the ascaristatic activity was determined by a modified Baldwin method (3).

EXPERIMENTAL

Part A (Lamson and Brown Method).—Fresh roundworms (*Ascaris lumbricoides, var. suis*) of the hog were obtained from a local slaughterhouse, and were handled according to the procedure advocated by Lamson and Brown. These worms, immersed in a normal saline bath maintained at 37.5°, were subjected to the action of an amount of each drug which would, if it all went into solution, make a 1–1000 concentration. Each bath was equipped with an electric stirring device. At specified times two worms were withdrawn from the bath, and after incubation for twenty hours at 37.5°, were then tested by dropping into water heated to 60° for viability. Sodium fluoride and hexylresorcinol were used as controls. The results are given in Table I.

In these tests, many of the compounds appeared as effective as sodium fluoride. The most striking results, however, were obtained with 3-(trifluoromethyl) phenol. This compound is a pale yellow, slightly water-soluble liquid, and is possessed of a penetrating odor. It is irritating to the skin. A saturated aqueous solution in 0.5-ml. doses, however, was nonlethal when injected via the tail vein into mice and young Wistar rats. Sloughing of the tails of mice indicated a local necrotic action. The intravenous LD_{50} (Karber method) as determined in Wistar rats (140 to 200 Gm.) using 50% propylene glycol as a solvent was found to be 57.25 ± 0.0349 mg./Kg. of body weight. The dose volume in each case was 0.5 ml. The sequence of events after the injections of lethal doses consisted of prostration, marked dyspnea, convulsions, and then death by respiratory failure within a minute after the introduction of the drug. The organs of these rats, examined grossly, did not show any abnormalities.

Since the effectiveness of 3-(trifluoromethyl)phe-

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TABLE I.—ASCARICIDAL ACTIVITY OF CERTAIN ORGANIC FLUORINE COMPOUNDS (LAMSON AND BROWN METHOD)

	Time of Exposure →	Minutes					Hours		
		2	5	10	15	30	45	1	2	3
		Mortality (Two Worms[a]), %								
1.	Perfluoro(methylnaphthalane)	0	0	0	0	0	0	0	0	0
2.	1,1,1-Trifluoropentachloropropane	0	50	50	50	0	0	50	0	50
3.	4-Hydroxy-4-(trifluoromethyl)-1,1,1,7,7,7-hexafluoroheptane	0	0	0	0	0	0	0	100	0
4.	1,2-Dichlorodecafluorocyclohexane	0	0	0	0	50	0	0	0	0
5.	3-(Trifluoromethyl)phenol	50	100	100	100	100	100	100	100	100
6.	4-Chloro(trifluoromethyl)benzene	0	0	0	0	0	0	100	100	100
7.	3-Chloro(trifluoromethyl)benzene	0	0	0	0	0	0	50	0	50
8.	2-Chloro(trifluoromethyl)benzene	0	0	0	50	0	100	50	50	50
9.	5-Chloro-1,3-bis(trifluoromethyl)benzene	0	0	0	50	0	50	0	50	100
10.	4-Chloro-1,3-bis(trifluoromethyl)benzene	0	0	50	100	0	0	0	0	0
11.	1,4-bis(Trifluoromethyl)benzene	0	50	50	50	50	0	0	50	0
12.	1,2-bis(Trifluoromethyl)benzene	0	0	0	100	0	100	50	0	0
13.	4-Methoxy-1,3-bis(trifluoromethyl)benzene	0	0	0	0	0	0	0	0	0
14.	Trichlorodifluoro(trifluoromethyl)benzene	0	0	0	0	0	0	0	0	0
15.	Perfluoroglutarimide	0	0	0	0	50	0	0	0	50
16.	Trifluoroacetanilide	50	0	50	100	50	50	0	50	50
17.	Sodium 2-fluorobenzoate	0	0	0	0	0	0	50	0	0
18.	Sodium 3-fluorobenzoate	100	0	50	100	50	0	0	0	0
19.	Sodium 4-(pentafluoroethyl)benzoate	0	0	0	0	50	0	0	0	0
20.	Ethyl 4-fluorobenzoate	0	50	50	0	50	0	50	50	0
21.	Allyl 4-(trifluoromethyl)benzoate	0	0	0	0	0	0	0	0	50
22.	Barium 4-fluorobenzene sulfonate	0	0	0	0	0	0	0	0	0
23.	Sodium 4-(trifluoromethyl)benzene sulfonate	50	0	0	0	0	0	0	0	0
24.	4,4′-Difluoro-diphenylsulfone	50	0	0	50	0	0	0	0	0
25.	Sodium fluoride (control)	50	0	0	0	0	0	0	50	50
26.	Hexyl resorcinol (control)	...	50	...	100	100	...	100	...	...

[a] The addition of propylene glycol (5% or 20%) or sodium glycocholate (0.1%) to the bath of further tests did not materially alter the results obtained with a saline bath.

nol approximates very closely the hexylresorcinol results, and since the organic fluorine compounds as a group do not, by this test, demonstrate outstanding ascaricidal activity, it would appear that the phenolic group of this compound is the major determining factor as far as ascaricidal action is concerned.

Part B (Modified Baldwin Method).—Most of the compounds studied in Part A were screened for ascaristatic activity using the Baldwin method. One-tenth gram of each drug was added to 100 cc. of bath. The bath temperature was maintained at 37.5° throughout the one-hour test. With santonin, used as a control, the tonus of the neuromuscular strip, after a temporary rise, fell sharply and remained at this low level throughout the test. During the rise in tonus, contractility ceased, but it reappeared for a brief period with the onset of reduced tonus. From this point on, contractility and rhythm were progressively decreased with complete cessation of contractility near the end of the one-hour period. 3-(Trifluoromethyl)phenol produced an immediate pronounced increase in tone with complete cessation of contractility. The segment appeared somewhat blanched. The heptane derivative (Table I, No. 3) caused, as a rule, little or no tonus changes with early (ten minutes) cessation of contractions. Perfluoroglutarimide caused almost immediate depression of contractility with some fall in tone. An occasional contraction was seen during the sixty-minute test. Trichlorodifluoro(trifluoromethyl)benzene and 3 chloro(trifluoromethyl)benzene gave similar results. The remainder of the compounds did not cause any significant changes in the activity of the neuromuscular segment of the roundworm as used in the Baldwin method.

CONCLUSIONS

1. Twenty-four organic fluorine compounds have been screened for ascaricidal and ascaristatic activity.

2. The results obtained with the Lamson and Brown method indicate that 3-trifluoromethylphenol is the most active ascaricide of the group of compounds studied.

3. Ascaristatic activity as indicated by the modified Baldwin method is noted for 4-hydroxy-4-trifluoromethyl-1,1,1,7,7,7-hexafluoroheptane. Perfluoroglutarimide, trichlorodifluoro(trifluoromethyl)benzene and 3-chloro(trifluoromethyl)-benzene were less effective.

REFERENCES

(1) Habermann, R., Enzie, F., and Foster, A., *Am. J. Vet. Research*, **6**, 131(1945).
(2) Lamson, P., and Brown, H., *Am. J. Hyg.*, **23**, 85(1936).
(3) Baldwin, E., *J. Parasitol.*, **35**, 89(1943).

Esters of 3-Nitrocumic Acid*

By ROSS M. BAXTER†, ‡ and P. A. FOOTE‡, §

Experimental data concerning a number of esters of 3-nitrocumic acid are presented. A number of physical constants are included. An attempt was made to prepare 3-hydroxy-2-carboxy-4-isopropylbenzoic acid.

ALTHOUGH terpene chemistry is one of the oldest branches of organic chemistry, its present-day importance continues to attract research workers. This is particularly true in the naval stores industry which is yielding a variety of commercial products including perfume materials. During the last twenty-five years the production of terpene products has been increased considerably in turpentining operations, from stumps, wood waste, and as by-products from kraft pulp mills.

During the last decade, more research has been applied to the monocyclic terpenes than the bicyclic terpenes with the result that this class of compounds has now acquired a distinguished place in industry. The structural relationship of the monocyclic terpene hydrocarbons to *p*-menthane and cymene can be readily seen. The conversion of these terpene hydrocarbons to cymene and the conversion of *p*-cymene to cymene acids is being carried out successfully on a commercial scale. One of these cymene acids is cumic acid. The fact that cumic acid (*p*-isopropyl benzoic acid) is a homolog of benzoic acid further increases its interest to the pharmaceutical chemist.

With these facts in mind the preparation of a number of esters of cumic acid and 3-nitrocumic acid was undertaken with a view to their possible value in flavors, cosmetics, and other pharmaceutical products. Another report from this laboratory is being submitted by Carkhuff and Gramling. It deals with esters of cumic acid whereas this report is limited to derivatives of 3-nitrocumic acid. 3-Nitrocumic acid has been prepared by several methods (1, 2).

The 3-nitrocumic acid was prepared using fuming nitric acid as the nitrating agent because the time involved in the nitration was relatively short (thirty to thirty-five minutes). The possibility of obtaining dinitro and trinitro derivatives was lessened. This method was further justified by higher yields and ease of purification. Ethyl alcohol was found to be the crystallizing solvent of choice. Using this method of nitration and one crystallization from ethyl alcohol, pure 3-nitrocumic acid was obtained in yields of ninety per cent.

The 3-nitrocuminoyl chloride was prepared by using two different reagents—thionyl chloride and phosphorous pentachloride. The reaction proceeded smoothly with both reagents. Refluxing was necessary in the case of thionyl chloride and when the reaction had gone to completion the excess thionyl chloride was removed under reduced pressure. An excess of phosphorous pentachloride was used after which the phosphorous trichloride was removed under reduced pressure. Attempts to purify the 3-nitrocuminoyl chloride by distillation at atmospheric pressure and at a pressure of 1 mm. failed. At atmospheric pressure decomposition was almost complete with the evolution of hydrogen chloride gas and the deposition of a black residue in the distilling flask. At a pressure of 1 mm., decomposition was also excessive and a loss of fifty per cent was experienced in attempting to purify the 3-nitrocuminoyl chloride in this manner. The method finally adopted was that of removing the excess reagent under reduced pressure, avoiding any further heating after the removal of the excess reagent. Using the 3-nitrocuminoyl chloride obtained in this manner, esters were prepared in good yields and purified.

Due to the difficulty in obtaining purified 3-nitrocuminoyl chloride, it was thought advisable to attempt esterification by other means also. Thus two other methods were used. Esterification was carried out in the presence of dry hydrogen chloride gas and in the presence of phosphorous trichloride.

With the exception of one ester, all the esters prepared were liquids. The one solid ester, the methyl ester, was purified by recrystallization from ethyl alcohol.

The liquid esters of 3-nitrocumic acid could not be purified by distillation at atmospheric pressure.

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‡ The authors wish to thank Newport Industries, Inc., Pensacola, Fla., for supplying the cumic acid for this study.
§ Dean of the College of Pharmacy, University of Florida, Gainesville.

All distillations of these liquid esters were carried out at pressures of 1–3 mm. Even at these pressures some decomposition was evident, especially when distilling esters of higher molecular weight. It seems evident from this work that the introduction of the nitro group in the aromatic nucleus decreases stability and increases the rate of decomposition in these compounds at elevated temperatures. At pressures of 1–3 mm., esters with boiling points above 160–165° showed definite signs of decomposition on distillation.

An attempt has been made to produce 3-hydroxy-2-carboxy-4-isopropylbenzoic acid. The possibilities of such a compound with respect to its properties as an antiseptic or insect repellent, as compared to cumic acid, was the reason for attempting this synthesis.

The synthesis of 3-hydroxy-2-carboxy-4-isopropylbenzoic acid involved the use of thymol as a starting material, the first step being the introduction of the carboxyl group in position 2, then oxidation of the 1-methyl to a carboxyl group.

The production of the *o*-thymotinic acid was carried out by the method (4), of dissolving the thymol in dry xylene, treating with metallic sodium and dry carbon dioxide at atmospheric pressure and at the temperature of refluxing xylene.

The oxidation of the 1-methyl group to a carboxyl was the next step in the synthesis. Three possible methods were available. In order to obtain the exclusive oxidation of the 1-methyl group, two methods were eliminated. The acid-permanganate oxidation results in the oxidation of both the methyl and the isopropyl groups. Alkaline permanganate oxidizes the methyl group almost exclusively but will also introduce a hydroxyl group in the isopropyl group, replacing the hydrogen on the tertiary carbon. Since the methyl group of *p*-cymene can be oxidized almost exclusively, to produce cumic acid, by catalytic air oxidation of *p*-cymene (3), it was thought advisable to try this method. The *o*-thymotinic acid was dissolved in benzene and the oxidation attempted by passing air through the solution in the presence of a catalyst consisting of equal parts of anhydrous manganese and lead acetates, two per cent by weight of the *o*-thymotinic acid. The air oxidation was allowed to proceed for seventy-two hours. At the end of this time the benzene was removed and the solid obtained was crystallized from ethyl alcohol twice. On checking the melting point and the neutral equivalent it was concluded that the product was *o*-thymotinic acid and that the oxidation had not taken place. Thus the compound 3-hydroxy-2-carboxy-4-isopropylbenzoic acid was not obtained.

EXPERIMENTAL

The cumic acid available was of technical grade and was purified by recrystallizing from methyl or ethyl alcohol. These two solvents proved the most satisfactory from the standpoint of yield and purity of the product.

3-Nitrocumic Acid.—Five-grams cumic acid (m. p. 116–117°) was dissolved in 15 Gm. concentrated sulfuric acid and cooled on ice. The mixture was stirred vigorously while 4 Gm. of fuming nitric acid was added slowly in small portions over a period of fifteen minutes. After thirty minutes from the start of the reaction, stirring was stopped and the reaction mixture was poured into ice water. The mixture was then stirred for a period of ten to fifteen minutes. The precipitate of 3-nitrocumic acid was collected on a suction filter and washed with distilled water until the filtrate was clear. The washed and dried 3-nitrocumic acid was crystallized once from ethyl alcohol. The yield of purified 3-nitrocumic acid (m. p. 156–157°) was 5.7 Gm.

3-Nitrocuminoyl Chloride.—Thirty-grams purified thionyl chloride was added to 50 Gm. of 3-nitrocumic acid. The mixture was refluxed on a water-bath for two hours and at the end of that time the solution was a clear red color. The excess thionyl chloride was removed at a pressure of 15 mm. The resulting liquid was placed in a vacuum desiccator over calcium chloride and dried under reduced pressure.

Attempts to purify this compound by distillation at a pressure of 1 mm. proved unsatisfactory as heating caused serious decomposition. The 49 Gm. of 3-nitrocuminoyl chloride, obtained from 50 Gm. of 3-nitrocumic acid, was used to produce esters and the impurities were removed in the purification of the esters.

Esters of 3-Nitrocumic Acid.—The methyl and thymyl esters were prepared by reacting together a molecular equivalent weight of 3-nitrocuminoyl chloride with molecular equivalent weights of methyl alcohol and thymol, respectively. The excess 3-nitrocuminoyl chloride was hydrolyzed with 5% aqueous potassium hydroxide. The residue was then washed with distilled water until neutral. The methyl ester was twice crystallized from ethyl alcohol. The liquid thymyl ester was purified by distillation at reduced pressure. The reaction in the case of the thymyl ester was carried out using benzene as a solvent.

The ethyl, *n*-propyl, isopropyl, *n*-butyl, *n*-amyl, and *n*-hexyl esters were prepared by passing dry hydrogen chloride gas into molecular equivalent weights of 3-nitrocumic acid and the alcohol. The reaction was carried out at the refluxing temperature of the alcohol and for a period of two hours. The excess 3-nitrocumic acid was removed as the potassium salt after which the esters were washed with distilled water until neutral. After drying over anhydrous sodium sulfate these esters were purified by distillation at reduced pressure.

The phenylethyl ester was prepared using molecular equivalent weights of 3-nitrocumic acid and phenylethyl alcohol with a slight excess of the calculated amount of phosphorous trichloride, and dry xylene as the solvent. The reaction was carried out at the temperature of refluxing xylene for a period of two hours. The xylene was then removed under reduced pressure and the residue was washed with

TABLE I.—CONSTANTS OF ESTERS OF 3-NITROCUMIC ACID

Ester of 3-Nitrocumic Acid	B.p.	M.p.	Refractive Index n^{25}	Ester Value: Calculated Theoretical	Ester Value: Average (3 Samples) Obtained
Methyl	....	64° (2)	....	247.7	245.3
Ethyl	291–292° (2), with decomp.	....	....	234.3	232.8
n-Propyl	149–150° at 1 mm.	....	1.5140	222.2	220.4
Isopropyl	147–148° at 1 mm.	....	1.5120	220.4	217.6
n-Butyl	165–166° at 1 mm.	....	1.5130	208.9	205.3
n-Amyl	181–182° at 2 mm.	....	1.5112	199.0	196.9
n-Hexyl	194–195° at 2.5 mm.	....	1.5074	189.1	186.2
Thymyl	196–198° at 1 mm.	....	1.5445	160.8	157.2
Phenylethyl	....	....	1.5310	....	....

5% aqueous potassium hydroxide, then with distilled water until neutral, and finally dried over anhydrous sodium sulfate.

SUMMARY AND CONCLUSIONS

The technical cumic acid was purified using several methods but the most satisfactory one was that of crystallizing the cumic acid from methyl or ethyl alcohol.

Three methods were used for the preparation of the 3-nitrocumic acid. The one of choice involved the use of fuming nitric acid as the nitrating agent.

Thionyl chloride was considered to be more satisfactory than phosphorus pentachloride for the production of the 3-nitrocuminoyl chloride.

The 3-nitrocuminoyl chloride obtained was used to prepare the methyl and thymyl esters with satisfactory yields. By passing dry hydrogen chloride gas through a refluxing mixture of the alcohol and the 3-nitrocumic acid, the following esters were prepared: ethyl, propyl, isopropyl, *n*-butyl, *n*-amyl, *n*-hexyl. The phenylethyl ester was prepared using phosphorous trichloride.

The following constants of these esters were found and recorded: boiling point, melting point, refractive index, and ester value. The esters prepared were all yellow, odorless, oily liquids, with the exception of the methyl ester which was a crystalline solid.

o-Thymotinic acid was prepared and air oxidation of the 1-methyl group to a carboxyl group, to produce 2-carboxy-3-hydroxycumic acid, was unsuccessful.

REFERENCES

(1) Widmann, O., *Ber.*, **15**, 2547(1882).
(2) Widmann, O., *ibid.*, **16**, 2567(1883).
(3) Palmer, C., *Ind. Eng. Chem.*, **34**, 1028(1942).
(4) Street, J. P., George, C. E., and Jannke, P. J., THIS JOURNAL, **37**, 180(1948).

WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Muscarine
Bulbocapnine
2-Hydroxy-5-methylazobenzene-4′-sulfonic acid
L-Mannose
L-Galactose
Naringenin
Phosphorylcholine
Phosphorylthiocholine
Vinyl iodide
Tridecamethylene bromohydrin
1,3-Dimethyl-1,3-diphenylcyclobutane
1,4-Dihydropyrazine-2,3-dione
1,2-Dihydropyridazine-3,4-dione
1,2-Dihydropyridazine-3,6-dione
Xanthotoxin
7-Hydroxy-1-H-v-triazolo-(d)-pyrimidine
7-(α)-12-(α)-Dihydroxyprogesterone
Sulfanylthiourea
Phloretin
Erythrophleine
O,N-Diacetylsalicylamide
Coniferyl aldehyde

Observations on the Toxicity of Methyl-Silicone*

By STANLEY F. KERN, ROBERT C. ANDERSON, and PAUL N. HARRIS

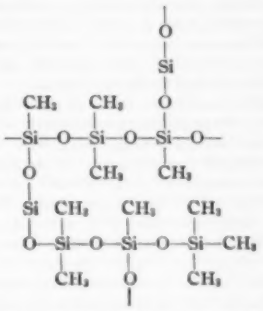
The results obtained by feeding rats silicone-coated food, and injecting silicone suspensions into mice and into rabbits are reported. The conclusion reached from the studies described is that the silicone examined possesses a low degree of toxicity.

THE DEVELOPMENT of various silicone products during recent years has resulted in their application in many systems where they come in contact with food and medicinal products. Applications of this sort are always open to question because of toxicological effects that may result from traces of such material contaminating the product. Because there was no information available on the toxicity of these materials, such an investigation was made.

Since Dri-Film No. 9977 appeared to meet our requirements, it was not deemed essential to study all types of silicones and silanes. The product resulting from polymerization of mixtures of methyl silanes of varying degrees of chlorination (G. E. Dri-Film No. 9977) was administered orally by coating the diet of animals, and by injection into various sites of a suspension of silicone particles in a dextrose-acacia solution. Although limited, these studies seemed adequate to answer any questions concerning the advisability of using this product in the manufacture of pharmaceuticals.

PREPARATION

Preparation of Coated Rat Food.—Five cubic centimeters of G. E. Dri-Film No. 9977 was slowly distilled from a small flask into an evacuated round-bottom flask containing five pounds of normal rat diet. The large flask was shaken vigorously during the distillation in order to insure homogeneous distribution of vapor on all particles. After the Dri-Film was completely distilled over and allowed to remain for about one minute, the flask was flushed by repeated evacuations to remove the HCl released in the polymerization. The presence of the silicone on the particles was confirmed by the difficulty encountered in wetting out the treated particles. The coating on the food was assumed to be nearly identical to the film formed on glass or ceramic surfaces by Dri-Film No. 9977. This film is most probably a 3-dimensional network of —Si—O— units with 1, 2, or 3 methyl groups attached to each silicone atom (1). For example:



Preparation of Suspension of Silicones for Injection.—Dextrose was coated in the same way as indicated above for the rat diet. In this case, however, 1 cc. of Dri-Film was used for every 50 Gm. of dextrose. The crystals after coating were difficult to wet out in water; but eventually water would penetrate the coating and dissolve the dextrose, leaving a film of silicone in suspension. In distilled water these films would agglomerate and were difficult to keep in suspension. The addition of acacia to the suspension stabilized it.

To 300 cc. of distilled water 0.715 Gm. of acacia was added. To 150 cc. of this solution 25 Gm. of dextrose coated with silicone was added, and the solution stirred in a Waring blender to disperse the silicone particles. To the other 150 cc. of solution 25 Gm. of untreated dextrose was added. Each of these solutions was filled into 1-cc. ampuls, sealed, and autoclaved. The solution as prepared had a *p*H of 5.5. The small flakes of silicone could be seen easily by observing the ampuls in a Tyndall beam.

RESULTS

Effect of Feeding Silicone-Coated Diet.—Five groups of 10 rats each were employed. The first group (control) received normal rat ration. The second group received a diet comprised of one part silicone-coated food and three parts normal food (Diet A). The diet of the third group consisted of equal parts of treated and untreated food (Diet B). The fourth group received a diet of three parts of coated and one part of normal food (Diet C). The diet of the fifth group consisted solely of the silicone-coated ration (Diet D). In this manner, the diets contained approximately 0.05, 0.1, 0.15, and 0.2% of silicone, respectively. The amount of food consumed by each rat was determined daily, and the body weight, hemoglobin, leucocyte count, and erythrocyte count of each rat were recorded weekly. During the course of the experiment, the food intake was found to be practically the same for all groups, and no significant differences in the body weights, hemoglobin, and blood cell counts of the animals on the different diets were observed, as

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Presented to the Scientific Section, A. PH. A., Jacksonville meeting, April, 1949.

shown in Table I. At the end of thirteen weeks the 50 animals were killed for histologic examination of their tissues, including heart, lungs, liver, spleen, stomach, intestines, kidneys, thymus, thyroid, and adrenals. No gross or microscopic abnormalities attributable to the treatment were found.

Effect of Injection of Silicone Suspension.—Five mice were given 13 intraperitoneal injections of 0.5 cc. of the silicone suspension over a period of seventeen days, and 5 mice received 13 intravenous injections of the same amount of material during the same period. None of these animals showed any recognizable reaction to the treatment.

Four rabbits each received, on one side of the body, 0.1 cc. of the suspension intracutaneously in 2 sites, 1.0 cc. subcutaneously, and 1.0 cc. intramuscularly. Control injections of the dextrose-acacia solution were made on the other side. One animal was sacrificed after twenty-four hours, the second after four days, the third after six days, and the fourth after eight days. No gross changes were evident at the sites of intracutaneous and subcutaneous injections. Hence, no sections could be made of the subcutaneous sites, but since the sites of the intracutaneous injections had been outlined in India ink it was possible to section them. One section of corium from the rabbit that was killed four days after inoculation included a focus of infiltration of small numbers of macrophages and polymorphonuclear leucocytes. The sections of skin from the other rabbits were normal. The site of the intramuscular injection in the control rabbit killed after one day could be detected because of slight hemorrhage, but in the remaining three could not be identified. In the muscles injected with the silicone suspension, the site was identified in the first rabbit by slight hemorrhage, and in two of the others, was manifested as a faint gray streak. Sections of the control and the silicone suspension injections into the first rabbit were similar, and showed necrosis of a few muscle fibers with slight macrophage infiltration. Sections were not made of the control injections in the other animals. Sections of the muscles of the other silicone-treated rabbits showed no reaction in one, and in two there was slight interstitial macrophage infiltration, with necrosis of a few muscle fibers in one of these.

TABLE I—EFFECT ON GROWTH AND HEMATOPOIETIC SYSTEM

Diet	Date	Av. Body Body Weight, Gm.	Av. Hemoglobin, %	Av. R. B. C. (millions)	Av. W. B. C. (thousands)
Control	4-15-48	61.3	110.1	6.25	10.47
	7-14	288.3	103.0	9.89	20.00
A	4-15	61.4	107.4	6.46	11.88
	7-14	288.7	102.7	9.12	20.78
B	4-15	62.0	99.1	5.98	8.75
	7-14	296.9	101.3	8.82	21.47
C	4-15	65.2	98.7	6.00	8.71
	7-14	313.0	100.2	9.01	21.41
D	4-15	64.2	93.0	6.56	7.96
	7-14	313.8	97.6	8.61	20.07

DISCUSSION

Since this paper was first prepared, a complete and comprehensive report on the toxicity of silicones and related compounds has been made by Rowe, Spencer, and Bass (2). The authors found silicone intermediates to be toxic. However, this would concern chiefly those interested in the manufacture of such intermediates. After testing commercial silicones by various procedures, they concluded that silicones as a class have a low toxicity and present no significant handling problem. This confirms our own conclusions with Dri-Film No. 9977.

SUMMARY

1. Administration to rats for thirteen weeks of silicone-coated food in amounts representing 25, 50, 75, and 100 per cent of their diets (silicone concentrations were 0.05, 0.1, 0.15, and 0.2 per cent, respectively) had no effect upon the body weight, hemoglobin, erythrocyte count, and leucocyte count. No gross or microscopic abnormalities were seen in the viscera.

2. Repeated intravenous and intraperitoneal injections into mice of a silicone suspension resulted in no demonstrable lesions.

3. Single intracutaneous, subcutaneous, and intramuscular injections into rabbits of the silicone suspension resulted in slight macrophage infiltration in one of eight intracutaneous injections; and three of four intramuscular injections resulted in slight macrophage infiltration and necrosis of a few muscle fibers.

REFERENCES

(1) Rochow, E. G., "Chemistry of Silicones," Wiley, 1946, pp. 45 ff. and 83 ff.

(2) Rowe, V. K., Spencer, H. C., and Bass, S. L., *J. Ind. Hyg. Toxicol.*, **30**, 332(1948).

Book Reviews

Medicine on the March. By MARGUERITE CLARK. Funk and Wagnalls Company, New York, 1949. x + 308 pp. 13 x 21 cm. Price $3.50.

Mrs. Clark has attempted to cover the march of medical progress during and since the five-year war period. The author believes "the book will have fulfilled its purpose if it (1) convinces the lay public of the magnitude of the medical research program now at work in the fight against disease; (2) enables the enlightened patient to talk more freely with his doctor and the doctor with his patient; (3) provides medical educators, public authorities, and hospital administrators with an up-to-the-minute medical résumé; and (4) gives editors, writers, radio commentators, and public speakers reliable information from professional sources as short cuts to more intensive research."

As for the usefulness of this book to the layman, the goal has been substantially reached. Mrs. Clark has done a good job of reporting. She gives evidence of having accurately reported what she has read and what people have said. She has also been conservative when the authors are conservative—there is little of the flashy and the ultradramatic.

Many will question, though, whether the interplay of facts and concepts with those of established medical opinion is truly represented here in a sufficiently authoritative manner for those trained in the medical sciences. In short, it does not seem logical that the author's third purpose can be attained by a book of this character. In the chapter, "New Drugs and Techniques," for example, the material bears considerable evidence of nonselectivity.

The reviewer found *Medicine on the March* to be interesting reading, nevertheless, and believes it to be a worth-while publication.

Drug Research and Development. Edited by AUSTIN SMITH and ARTHUR D. HERRICK. Revere Publishing Company, New York, 1948. xi + 596 pp. 14.5 x 23 cm. Price $10.

The essence of modern developmental research in the drug field is teamwork and collaboration. The drug manufacturer, the distributor, the research and laboratory worker, the advertising counsel and sales representative, the physician, and the pharmacist, all participate in some fashion in such a developmental program.

It is because of the teamwork nature of developmental research that each man must know exactly where his responsibility and authority lie and he must know enough about the other fellow's job to be able to see his point of view and adjust his own viewpoint to accomplish the task.

Drug Research and Development sets out to promote this understanding and to present the necessary background. Because of the careful selection of authors, who are dealing with these problems in their daily work, the aims are accomplished in a most satisfactory and practical manner.

Each author presents his aspect of the problem in broad retrospect, giving sufficient detail to clinch the point, without subjecting the reader to a mass of less pertinent information. The editors have quite evidently given sufficient leeway to the authors to assure a complete expression of their opinion. At the same time the various phases have been pulled together in such a way that the central concept is maintained and duplication is at a minimum.

Every category of drug products, both the so-called ethical products and the proprietaries are included and such phases as chemical, pharmacological and biological research, clinical testing, development of facilities and controls, labels and labeling, packaging and storage, patent and trade-mark law, drug regulation, biologic regulation, new drug applications, drug certification, Council acceptance, promotion and marketing, advertising, "detailing" the physician, professional acceptance, and retail price maintenance are covered. The experts covering these various areas are Austin Smith, Edgar B. Carter Windsor C. Cutting, Walter J. Derenberg, Isaac W. Digges, Charles S. Downs, Harvey B. Haag, Robert A. Harđt, Arthur D. Herrick, Thomas H. Jones, Charles R. Linegar, Amel R. Menotti, John G. Searle, Maurice L. Tainter, Edward Thomas, Walton Van Winkle, Jr., and Walter E. Ward.

Natural Products Related to Phenanthrene. By LOUIS F. FIESER and MARY FIESER. 3rd ed. Reinhold Publishing Co., New York, 1947. xii + 704 pp. Price $10.

Usually when a book has gone into a third edition, changes are so minor that nothing much can be said by the reviewer. This reference book on the phenanthrenes is an exception, for the authors have nearly rewritten the book. The outlook on steroid chemistry has changed considerably since the advent of the second edition, and the authors have been meticulous about entering new concepts without destroying the best of the old.

A few of the old chapters (carcinogenic hydrocarbons and triterpenoid sapogenins) have been omitted entirely and new chapters on the adrenal cortical hormones, steroid metabolism, etc., have taken their place. A lengthy chapter on the sterochemistry of the steroids by Richard B. Turner adds much to the book.

To those not working in this field, steroid chemistry is difficult, yet the authors have made it appear to be easy, because of the lucid, free-flowing style of their writing.

There are so many compounds of pharmaceutical interest in this group, i.e., bile acids, vitamin D, sex hormones, adrenal cortical hormones, cardiac glycosides, and the steroid and terpenoid alkaloids, that no scientific pharmaceutical reference library is complete without this book.

The book is well indexed with both an author and a subject index. The many charts are excellent and the line drawings of the formulas are unusually clear and neat.

Biochemical Preparations. Volume I. HERBERT E. CARTER, Editor-in-chief. John Wiley and Sons, New York, 1949. xi + 76 pp. 14.5 x 23 cm. Price $2.50.

Biochemical Preparations is a new publication to augment *Organic Syntheses* and *Inorganic Syntheses* by giving precise and full directions for preparing substances used in biochemistry. Such publications are invaluable to the busy research biochemist.

The value of this publication lies in the fact that, before publication, each preparation is carefully checked in a laboratory other than that with which the original submitter is associated. Isolation methods are emphasized but if the best results are obtained by synthetic methods, the synthetic method is published. The format is similar to that of *Organic Syntheses*. A table of compounds of biochemical interest which have appeared in *Organic Syntheses* (through Volume 27) is a helpful addition.

Compounds covered in the first volume of this projected series are adenosine diphosphate and triphosphate, *l*-alanine, *l*-serine, azobenzene-*p*-sulfonic acid trihydrate, *p*-hydroxyazobenzene-*p*-sulfonic acid, 5-nitronaphthalene-*l*-sulfonic acid dihydrate, casein, *p*-3,4-dihydroxyphenyl-*l*-alanine, diphosphopyridine nucleotide, the α-glucose-*l*-phosphates, *l*-glutamine, *dl*-glyceraldehyde-3-phosphoric acid, lycopene, *l*-lysine monohydrochloride, lysozyme, and *d*-tyrosine.

1950 Iodine Research Award Nominations Requested

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1950 Iodine Educational Bureau Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine. Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1950 Award, nominations must be received on or before January 1, 1950.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During a period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Iodine Educational Bureau, Inc.

The award consists of $1000 and a diploma setting forth the reasons for selection of the recipient. It may be presented annually at the annual meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. In addition to the sum of the award, the recipient will receive an allowance of not more than $250 to defray his expenses in attending the meeting.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council and which functions under prescribed rules. The present committee includes Justin L. Powers, chairman; Louis Gershenfeld, Charles O. Wilson, Harvey B. Haag, Heber W. Youngken, John C. Krantz, and Frank O. Taylor.

The award is now in its third year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist. Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, will be the recipient of the second award and will give the 1950 Iodine Award lecture.

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